University of Alberta

Examining the Effect of Dietary Fructose Fed During Suckling on Glucose Metabolism in the Rat at 18 Days

By

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

in

Nutrition and Metabolism

Department of Agricultural, Food & Nutritional Science

Edmonton, Alberta

Spring 2005

0-494-08122-8

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**The challenge of being human is so great
that no one can get it right all the time.**

--Rabbi Harold S. Kushner

People travel to wonder at the height of mountains, at the huge waves of the sea, at the long courses of rivers, at the vast compass of the ocean, at the circular motion of the stars; and they pass by themselves without wondering.

--St. Augustine of Hippo

I no doubt deserved my enemies, but I don't believe I deserved my friends.

--Walt Whitman

This is dedicated to all who inspire others to act with courage while fostering dignity and respect.

ABSTRACT

The purpose of this study was to examine the effects of dietary fructose (FR) fed during suckling on glucose metabolism before weaning. Rat pups were fed either a rat milk substitute (RMS) or RMS containing FR via a gastrostomy tube from day 7 to day 18 and were compared to mother fed (MF) controls. Oral glucose tolerance tests (OGTTs), insulin secretion and *in vitro* β-cell characterization studies, pancreatic morphology, circulating plasma lipid concentrations and fatty acid synthase (FAS) activities were performed. After 11 days of dietary FR, plasma insulin concentrations during the OGTT were more than two-fold higher compared to RMS and MF rats at both 15 and 30 minutes, but did not differ at baseline or at 45, 60 and 90 minutes. During the OGTT, plasma glucose concentrations of FR fed rats were lower than both MF and RMS rats at 30, 45 and 60 minutes. However, no significant differences were observed in the insulin response to glucose *in vitro,* or in morphological studies (β–cell fraction (%), β–cell mass, or β–cell area) among the three diet groups. There were no significant differences in plasma triglyceride concentrations, whereas plasma non-esterified fatty acid (NEFA) concentrations in FR and MF rats were higher compared to RMS rats. FAS activities in hepactocytes isolated from MF rats were almost twice as high as those observed in FR and RMS rats. Therefore, FR introduced during suckling appears to affect insulin secretion *in vivo* at 18 days of age. This suggests that the various aspects involved in the development of glucose and lipid metabolism are sensitive to dietary carbohydrate intake during suckling. This study lays the foundation for the investigation of the long term effects of dietary FR during suckling.

ACKNOWLEDGEMENTS

The completion of this thesis would not have been possible without the generosity, patience and love of the people who supported me on this journey.

To Dr. R.C Bell, thank you for giving me the opportunity to appreciate the art of science and the discipline, creativity and practice that it requires. I am also grateful for your generosity.

I would like to acknowledge the support of my supervisory committee, Dr. C.J. Field and Dr. G.S. Korbutt, thank you for your patience.

Many thanks to Abha Hoedl, Joan Turchinsky, Donna Taylor, Dr. Y.K Goh, Minh Huynh, Desmond Pink and Kathryn Proudfoot. Without the coffee breaks and the combination of your help, friendship and sense of humor there would be more tears than sweat and blood.

Thank you to the technical support of Max Amerongen, Jody Forslund and Francine Hodder. Without your spirit of cooperation and service these hoops could not have been jumped.

I would also like to recognize generous contribution of Dr. G.S. Korbutt and his highly skilled employees especially, James Lyon, Lynette Elder and Jennifer O'Hara.

Thank you to my friends Suzanne Frison, Gord Grant, Eek Joong Park and Suh Young Park and Vanesssa Lien. You extended your hearts and homes to me and carried me through the trials. Thanks for the laughter and karaoke.

Thank you to Fr. David Bittner, Fr. Tim Scott and Fr. Tim Boyle for their prayers, advice and guidance. Thank you for reminding me about nourishment.

I would like to thank my parents, Les and Mary and my sister Naomi and brother Eric, without your love and support none of this would be possible.

This work was funded by grants from NSERC and the Canadian Diabetes Association.

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LIST OF ABBREVIATIONS

ACC.....	acetyl Co-A carboxylase
AP.....	arterial blood pressure
AR.....	artificial rearing
ATP.....	adenosine triphosphate
BCA.....	bicinchoninic acid protein
BCF.....	β-cell fraction
BMI.....	body mass index
BIA.....	bioelectrical impedance analysis
BrdU.....	bromo-deoxyuridine
CHD.....	coronary heart disease
DEXA.....	dual-energy X-ray absorptiometry
FAS.....	fatty acid synthase
FK.....	fructokinase
FFA.....	free fatty acids
FFM.....	fat free mass
FR.....	fructose
G6PD.....	glucose-6-phosphate dehydrogenase
GIR.....	glucose infusion rate
GLP.....	glucagon-like peptide
HC.....	high carbohydrate
HG.....	high glucose
HGO.....	hepatic glucose output
HF.....	high fat
HFR.....	high fructose
HFCS.....	high fructose corn syrup
HS.....	high sucrose
IGT.....	impaired glucose tolerance
IVGTT.....	intravenous glucose tolerance test
HDL.....	high density lipoprotein
LBM.....	lean body mass
LDL.....	low density lipoprotein
LP.....	low protein
ME.....	malic enzyme
MF.....	mother fed

NEFA.....	non-esterified fatty acids
OGTT.....	oral glucose tolerance test
PCNA.....	proliferating cell nuclear antigen
PFK.....	phosphofructokinase
RMS.....	rats' milk substitute
SI.....	stimulation index
T1D.....	Type 1 diabetes
T2D.....	Type 2 diabetes
TG.....	triglyceride

CHAPTER 1. LITERATURE REVIEW

1. Introduction to Type 2 Diabetes Mellitus

Diabetes mellitus is defined as "a metabolic disorder characterized by the presence of hyperglycaemia due to defective insulin secretion, insulin action or both (Canadian Diabetes Association, 2003)." Approximately 1.1 million Canadians aged 18 years or older have diabetes (2000/01 Canadian Community Health Survey; Young & Millar, 2003). The majority of cases of diabetes fall into two major categories. Type 1 diabetes (T1D) is the result of an absolute impairment in insulin secretion due to islet β-cell destruction that is mediated by an autoimmune process. Type 2 diabetes (T2D) is a heterogeneous condition that results from the combination of insulin resistance and may be accompanied by a defect in insulin secretion. People with T2D may remain asymptomatic or undiagnosed for years. T2D is more common than T1D as it affects approximately ninety percent of people who are living with diabetes (Canadian Diabetes Association, 2003). In 1998, the total economic burden of diabetes in Canada was between $4.8 and $5.2 billion dollars (US dollars) (Dawson et al, 2002). Diabetes mellitus is the 7th leading cause of death (all-cause mortality) in the US population (Rosenberg et al, 1996).

Syndrome X as was first defined as "the cluster of abnormalities occurring in non-diabetic persons at increased risk for coronary artery disease (Reaven, 1992)." Its components include some degree of glucose intolerance, hypertriglyceridemia, low HDL-cholesterol concentration and an increase in blood pressure. Syndrome X is now known as metabolic syndrome and its diagnosis requires three or more of the following: elevated

plasma glucose (>6.1 mM), elevated blood pressure (>130/85 mm Hg), elevated triglycerides (>1.7 mM), low HDL cholesterol (<1.0 mM for men; <1.3 mM for women) and abdominal obesity (>102 cm for men and >88 cm for women). Based on these criteria, an estimated 43.5% of the US population over the age of 50 has Metabolic Syndrome (Alexander et al, 2003) and approximately 4% of American adolescents meet this criteria for the diagnosis of metabolic syndrome (Cook et al, 2003).

Diabetes and the metabolic syndrome contribute largely to the burden of "non-communicable conditions" (Last, 1998). Diabetes is "associated with significant long-term sequelae, particularly damage, dysfunction and failure of various organs especially the kidney, eye, nerves, heart and blood vessels (Metzler et al, 1998)." Metabolic syndrome can also contribute to significant long-term sequelae independent of diabetes. Even small increases in blood glucose concentration, including glucose intolerance or impaired fasting glucose can lead to an increased risk for cardiovascular mortality. Alexander et al (2003) reported that people over the age of 50 who had metabolic syndrome without diabetes had a 5.2% higher prevalence of coronary heart disease compared to those without the metabolic syndrome. Therefore, it is important to understand the factors that lead to the development of these conditions if we hope to curb their incidence in the future.

Results from interventions targeted at preventing T2D in adults at high risk for the disease have recently been published. The largest prevention trial to date is the Diabetes Prevention Program (DPP) (Diabetes Prevention Program Research Group, 2002; Palmer et al, 2004). The DPP randomized 3,234 overweight patients aged 25 years and older (20% older than 60) with impaired glucose tolerance (IGT) to three treatment groups.

These patients received intensive lifestyle counseling (ILC), standard lifestyle advice plus metformin, or standard lifestyle advice plus placebo and a comparison was done on the effects of the three treatments on the progression from IGT to T2D (Knowler et al, 2002; Palmer et al, 2003). Subjects in the ILC group received individual counseling to reach a goal of 7% weight loss and >150 min/wk of moderate intensity exercise. After 2.8 years, subjects in the ILC group had a 58% reduction in T2D risk compared to control subjects; whereas subjects receiving metformin and general lifestyle recommendations had a 31% reduction in T2D risk. This study showed that ILC is effective at preventing or delaying the progression to T2D.

One question that arises from the DPP is at what age is it appropriate to begin targeting the prevention of chronic disease? Should programs wait until adulthood or could interventions occur at an earlier age? Until recently, little attention was paid to the effects of nutrition during early periods of development on chronic diseases later in life.

2. Metabolic Imprinting: Long-term Consequences of Early Diet

An emerging doctrine in nutrition science examines the relationship between early nutritional intake (foetal, neonatal, infant, childhood and adolescent) and later chronic disease. This concept has been incorporated into three different, but related, hypotheses: thrifty phenotype hypothesis; foetal origins hypothesis or Barker hypothesis; and metabolic imprinting. Hales & Barker (1992) proposed the "thrifty phenotype" hypothesis (Figure 1) to explain how "poor nutrition in foetal and early infant life [may be] detrimental to the development and function of beta (β) cells of the islets of

Langerhans," And predispose to the later development of T2D." This hypothesis was formulated using observations from the Hertfordshire cohort.

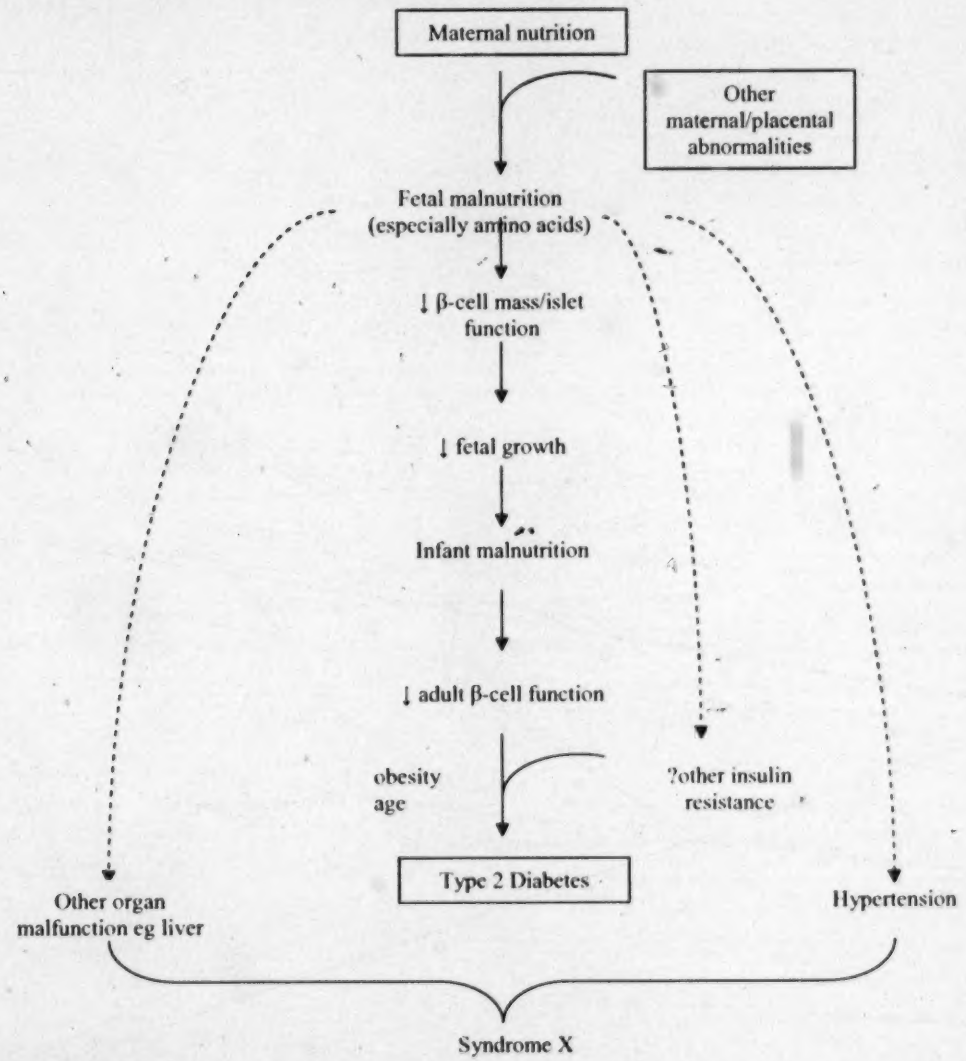


Figure 1. Key features of the "thrifty phenotype" hypothesis of the etiology of T2D. (Adapted from Hales & Barker, 1992)

Barker & Osmond (1986) showed that rates of death from cardiovascular disease in adulthood were higher in less affluent regions of England and Wales that had high rates of infant mortality 70 years earlier. Since there was an association between birth weight and neonatal mortality, they suggested that perhaps impaired foetal growth was also associated with cardiovascular disease. Further investigation using this cohort led to similar findings with: ischemic heart disease (Barker et al, 1989); T2D (Barker et al, 1991); and risk factors for coronary heart disease, including high blood pressure, abnormalities in lipid metabolism and blood coagulation later in life (Barker, 1995). Based on these observations, Barker (1995) expanded on the thrifty phenotype hypothesis and proposed the "foetal origins hypothesis" to suggest that "poor nutrition, health, and development among girls and young women is the origin of high death rates from cardiovascular disease in the next generation…[Whereby] the foetus responds to under-nutrition with permanent changes in its physiology and metabolism and these lead to coronary heart disease and stroke in adult life (Figure 2)." Further, it is possible that the effects of foetal under-nutrition vary depending on which trimester of pregnancy the nutritional insult originated.

The foetal origins hypothesis (Barker, 1995) expanded on a mechanistic theory proposed by Lucas (1991). This relates to the idea that major diseases occurring later in life may, in part, be a result of permanent alterations in structure, physiology and metabolism that are "imprinted" during foetal development (Godfrey & Barker, 2000; Waterland & Garza, 1999; Patel & Srinivasan, 2002). Lucas (1991) defined Metabolic Programming as a "phenomenon whereby a nutritional stress/stimulus applied during critical periods of early development permanently alters an organism's physiology and

metabolism, the consequences of which are observed much later in life in the absence of the stress/stimulus that initiated them." Waterland & Garza (1999) suggested that Metabolic Programming be termed "imprinting". The terms are currently used interchangeably in the literature. Waterland & Garza (1999) proposed that for metabolic imprinting to occur, there must be: 1) a susceptibility limited to a critical ontogenetic window early in development; 2) a persistent effect lasting through adulthood; 3) a specific and measurable outcome (that may differ quantitatively among individuals); and 4) a dose response or threshold relation between a specific exposure and outcome.

According to Stein et al (2002) "the literature relating birth weight (as a proxy indicator for foetal nutritional status) to risk factors for cardiovascular disease, especially blood pressure level, disruption of the glucose-insulin regulatory axis and metabolic syndrome has become voluminous." It has been proposed that "retarded intrauterine growth" leads to adult hypertension. Growth retardation has been defined simply in terms of anthropometric measures at birth, for example, birth weight, birth length, ponderal index (birth weight/birth length^3) and crown-heel length (Leon et al, 1996). The discussion will be limited to the association between birth weight and birth length and subsequent risk for adult chronic disease.

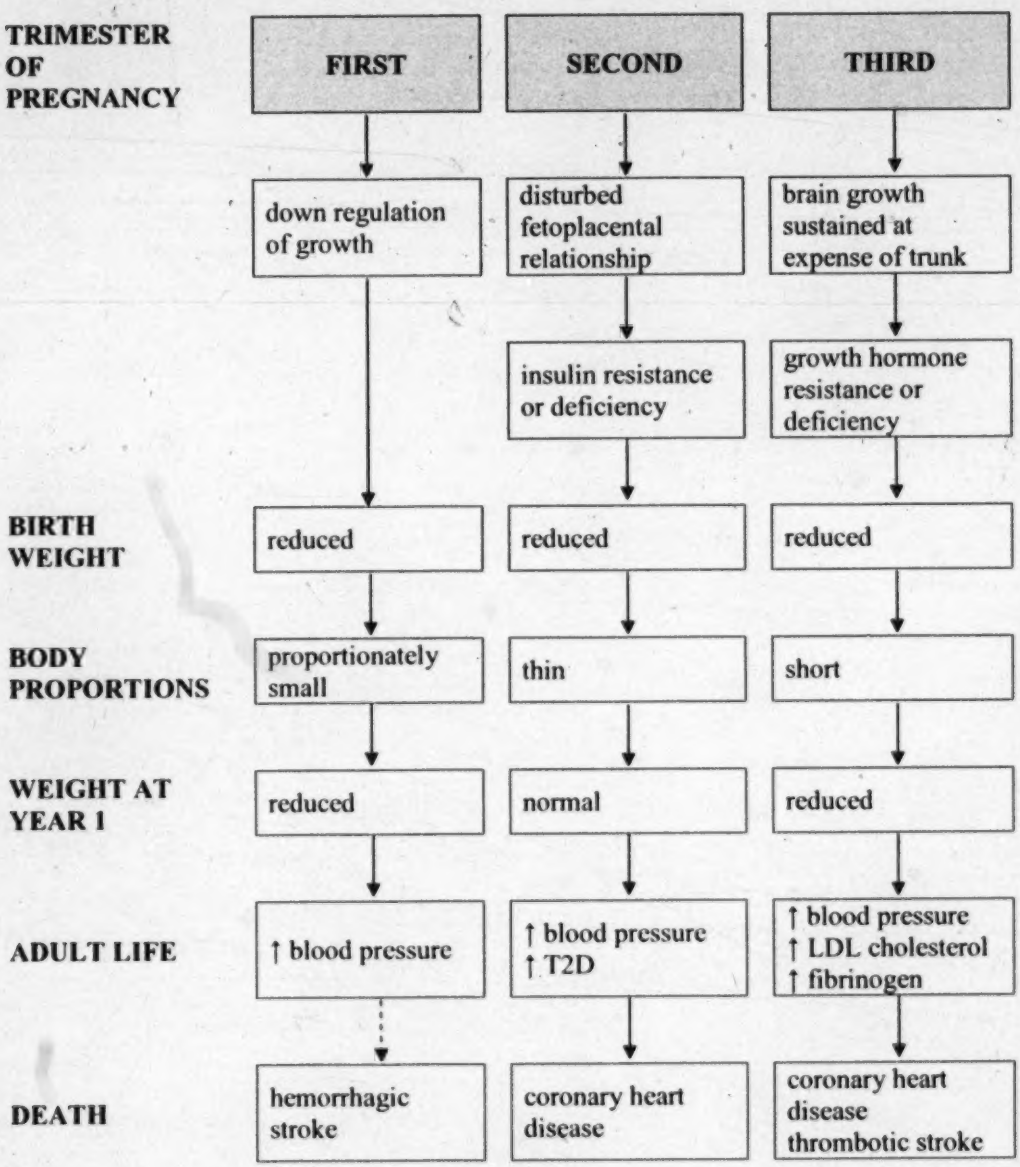


Figure 2. Framework of ideas in the fetal origins hypothesis linking fetal under-nutrition with later abnormalities (taken from Barker, 1995)

2.1 Metabolic Imprinting during the Foetal Period

2.1.1 The Association Between Low Birth Weight and Risk for T2D in Humans

Studies examining the relationship between low birth weight and risk for T2D in humans have been conducted in many populations (Stein et al, 1996; Vestbo et al, 1996; Wilkin et al, 2002; Skidmore et al, 2004). The largest study (70 297 female participants) was the Nurses Health Study in the United States (Rich-Edwards et al, 1997). The study found that increased birth weight was inversely associated with decreased risk for non-fatal cardiovascular disease. Birth weight was also inversely associated with adult body mass index (weight (kg)/height (m^2);BMI), adult hypertension, cholesterol concentrations and incidence of T2D. In addition, low birth weight (<2 495 grams) was associated with a 23% increase in non-fatal cardiovascular disease compared to all other women (Rich-Edwards et al, 1997).

Several studies have been conducted that examine the relationship between birth weight and the prevalence of risk factors relating to T2D or cardiovascular disease such as decreased insulin sensitivity or high blood pressure. Leon et al (1996) studied the relationship between birth weight and blood pressure at 50 years of age among Swedish men. They separated subjects into quartiles based on birth weight (<3 250g; 3 250-3 750g; 3 750-<4 250g; and ≥4 250g). They found that subjects from the lowest two birth weight groups had higher systolic blood pressure as adults. Also, birth weight was positively correlated with BMI at age 50. As BMI increased, the association of birth weight with blood pressure became stronger. The largest effect of birth weight on blood pressure was in the group of men born at term. At age 50 they were above median height and in the highest third of BMI, a 1000 g increase in birth weight was associated with a

change of -15.9 mm Hg (-26.5 to -5.2 mm Hg) systolic and -7.4 mm Hg (-14.4 to -0.4 mm Hg) diastolic blood pressure. This suggests that there is an interaction between birth weight and adult weight in the development of hypertension.

Using the same cohort, Lithell et al (1996) studied the association between birth weight and insulin resistance at 50 years of age using an intravenous glucose tolerance test (IVGTT). Subjects were separated into groups using the same birth weight cut-offs as Leon et al (1996) and by adult weight according to BMI (<23.55; 23.55-25.99; and ≥26). Subjects who were in the lowest quartile of birth weight (<3 250g) and who were in the highest BMI category had the highest fasting insulin levels and higher insulin concentrations at 1 hour after the intravenous glucose bolus. This suggested an inverse association between birthweight and insulin resistance (Lithell et al, 1996). They further investigated the relationship between birth weight and incidence of T2D at 60 years of age and found an inverse relationship between birth weight and incidence of T2D. However, after including fasting and 60-minute insulin concentrations in a multiple regression model along with body mass index, the association between T2D and low birth weight was no longer significant. In Pima Indians (aboriginal Americans from the Southwestern United States) low birth weight has also been shown to be associated with increased risk for T2D (McCance et al, 1994).

Skidmore et al (2004) studied the relationship between birth weight and lipid concentrations in a birth cohort from the United Kingdom. There appeared to be no interaction between birth weight and cholesterol levels in women. However, in men a 1 kg increase in birth weight was associated with a reduction (-0.15mmol/L) in total plasma cholesterol concentration and was strongest among men who had higher BMIs at age 53

years. Therefore, having a relatively higher birth weight was protective against having high cholesterol in this cohort., Stein et al (1996) observed 20% higher incidence of coronary heart disease among people born in South India who weighed less than 5.5 lbs (2 500g) at birth and no incidence of coronary heart disease among those who weighed more than 6.5 lbs (2 955 g) at birth. Studies relating birth weight to risk for chronic disease in adulthood are limited since they are unable to control for factors during adolescence or adulthood that may also contribute to the development of disease.

Stein et al (2002) investigated whether birth weight is associated with risk factors for chronic disease in Guatemalan adults at 24 years of age. The authors found that in women, birth weight was positively associated with adult BMI but there was no association in men. Birth weight was positively associated with both systolic and diastolic blood pressure in women but was not associated with plasma glucose concentrations or total plasma cholesterol, low density lipoprotein, high density lipoprotein, triglyceride (TG) concentrations). While there was no association between birth weight and adult BMI, TG concentrations, blood pressure or glucose levels; plasma concentrations of total cholesterol and LDL cholesterol were inversely related to birth weight in men (Stein et al, 2002). For both men and women these relationships were strongest in the highest tertiles of birth weight. However, adults in the highest tertiles of BMI (>22.8 for men; >24.2 for women) had a worsening of their cardiovascular disease risk profiles.

Stein et al (2002) were unable to observe fetal programming in their Guatemalan cohort. However, there may other factors to consider about this population before making this conclusion, for example they concluded that adult BMI was the greatest predictor of

increased CVD risk. But the high tertiles of BMI were still quite lean and were on average less than 25. Still, reduced foetal growth may be associated with changes in metabolism that lead to risk for adult chronic diseases (ie. plasma lipid concentrations). However, since adult BMI more significantly influenced these parameters, it is important to consider that factors present during the postnatal period that contribute to adult BMI may exacerbate this risk.

Barker (1995) hypothesized that different phenotypes would result from under-nutrition depending on the stage of gestation of the nutrition insult. For example, thinness at birth may be the result of mid-to-late gestation under-nutrition; whereas, shortness at birth may be the result of limited linear growth and possibly a brain-sparing mechanism of a nutritional insult sustained throughout pregnancy (Barker et al, 1993; Flanagan et al, 2000). Literature relating shortness at birth with chronic disease risk in adulthood is not as extensive as birth weight studies. However, birth length has been studied as another indicator of foetal growth and has been used in the investigations of the association between birth size and chronic disease.

Stein et al (1996) examined the relationship between birth length (≤18 inches; 18.1-19 inches; >19 inches) and risk for cardiovascular disease. They found that short length was associated with increased risk for cardiovascular disease at all birth weights. Flanagan et al (2000) studied the relationship between foetal growth and insulin resistance and sensitivity at 20 years of age in an Australian cohort. They found that in men, short birth length was associated with an increased first phase insulin response to glucose (AIRglc), increased glucose effectiveness and insulin resistance (Flanagan et al, 2000).

Retrospective epidemiological studies are limited in their ability to determine causation, although they clearly identify associations between small size at birth and later T2D. Many factors are active during pregnancy and may have an influence on foetal growth, including but not limited to: maternal nutrient intake; weight gain; smoking; circulating plasma hormone levels; placental development; maternal hypertension. Data from the Dutch Hunger Winter (1944-1945) suggest that lower birth weight is associated with maternal food restriction during the third trimester; while birth weight is not affected by nutritional restriction during the first trimester (Stein et al, 1995). In future studies it may be important to consider how the early nutritional environment impacts the development of body composition and its different compartments (ie. fat vs fat free mass). For example, Birth weight may be confounded by differences in body composition (eg. a heavier baby may have more lean body mass and may not have the same risk as a lighter baby with more fat mass).

2.1.2 Other Aspects of Growth May Be Associated with Risk for T2D in Humans

It is possible that the associations between birth weight or birth length and risk for chronic disease is confounded because foetal growth may be related to other factors known to affect metabolism. Several researchers have studied the relationship between birth size and lean body mass (LBM) in childhood, adolescence (Singhal et al, 2003) and adulthood (Phillips et al, 1995). Singhal et al (2003) conducted a study of two different cohorts of children. The cohort included 78 adolescents aged 13-16 years and body composition was measured using bioelectrical impedance analysis (BIA). The second

cohort was a group of children (mean age 7.4 years) and body composition was measured using using dual-energy X-ray absorptiometry (DEXA).

Singhal et al (2003) reported that higher birth weight (1 SD above mean) was significantly associated with a 2-3% increase in fat-free mass in adolescents for both sexes. This association was independent of age, sex, height, pubertal stage, socioeconomic status, and physical activity and similar observations were found in the younger children. This study suggests that higher FFM may be associated with decreased risk for chronic disease rather than high fat mass being associated with increased risk for chronic disease. Similar studies relating birth size to later body composition have been conducted in older adults (Phillips, 1995; Gale et al, 2001; Aihie Sayer et al, 2004). Gale et al (2000) compared birth size to body composition (DEXA) at 70-75 years of age. The authors reported that in both sexes, birth weight was positively associated with lean body mass after adjusting for age, sex, height and weight. While not statistically significant, there was a trend for whole body fat mass to be greater among subjects who were in the lowest third of birth weight. Aihie Sayer et al (2004) conducted a similar study using male subjects (mean 64 years of age) from the Hertfordshire cohort. In their study FFM and fat mass were measured using skin-fold thickness, which is a less precise measurement than either BIA or DEXA. They found a strong, positive correlation between birth weight and FFM. Birth weight, adult BMI and waist circumference were also positively correlated, but this relationship was less strong. The association between foetal growth and later body composition may add another dimension to the foetal programming hypothesis. Since muscle is an important site for insulin stimulated glucose

uptake, 'programming' of a smaller proportion of FFM by low birth weight could adversely affect insulin sensitivity.

It is not possible to examine maternal nutrient intake as a cause of poor foetal growth and subsequent development of T2D in adulthood in humans in an experimental situation since purposefully reducing a mother's diet to investigate differences in foetal growth is unethical (Symonds et al, 2000). Therefore, animal models are necessary to explore questions about the mechanisms of metabolic imprinting.

2.1.3 Animal Models of Foetal Malnutrition in the Rat

There are several animal models that have been used to examine the effects of maternal nutrient intake during pregnancy on foetal growth and development and subsequent risk of disease. The most extensively studied model involved feeding rat dams an isocaloric, low protein (LP) diet during the entire course of gestation. Another model reduces total energy intake to approximately 50% of ad libitum diet. There are also models in which a surgical procedure is used to restrict total blood flow to the foetus usually for the final 48-72 hours of gestation. Since the LP model has been most extensively studied, this discussion will concentrate on results using this method.

The LP model involves feeding dams a diet containing eight versus 20% kilocalories from protein during gestation or gestation and lactation with no changes in total energy. It results in a litter size within the normal range (8-12 pups) and although pups are of lower body weight they appear healthy (no congenital defects), they reach developmental milestones (eg. eyes opening) and the mother is capable of nursing throughout lactation (Snoek et al, 1990; Dahri et al, 1991; Desai et al, 1995). Snoeck et al

(1990) demonstrated that at 21.5 days gestation, offspring of LP mothers had reduced body weight and differences in the endocrine pancreatic structure (β-cell proliferation, islet size, vascularization) and a reduction in pancreatic insulin content.

Petrik et al (1999) used the LP model to examine how the balance of islet cell replication and survival in the endocrine pancreas is altered in offspring before birth and in infancy. This study focused exclusively on female pups at the end of gestation (day 19.5 to 21.5 days) or born and raised in litters (eight pups) that were suckled by control dams and studied postnatally (between 2 and 21 days of age). Since the insulin-like growth factor (IGF) axis has been shown to be responsive to nutrition (Estivariz et al, 1997; Thissen et al, 1994) they also examined if alterations in pancreatic expression of IGF-I, or IGF-II were associated with cell proliferation and/or apoptosis, or programmed cell death.

Offspring of LP mothers weighed approximately 8% less than control (C) rats at birth? and at 21 days of age these offspring weighed 33% less than control rats. While no differences in pancreatic weight were detected at birth, after day 14 the weight of the pancreas was 46% less in offspring from LP fed dams than the control dams. Although neither blood glucose concentrations nor plasma insulin concentrations varied between the two groups at any age, the mean islet area was reduced by more than 50% in LP pups compared with C pups of the same age. At postnatal day 14 the mean islet area was reduced by 70%. Further classification of cell types within the islet showed that β-cells (stained positive for immunoreactive insulin) made up a smaller area of the islet in LP pups compared to C pups at all ages, whereas α-cells (stained positive for immunoreactive glucagon) constituted a larger area in pancreata of LP offspring.

Petrik et al (1999) measured whole pancreas IGF-I and IGF-II mRNA using Northern blot hybridization and compared sites of IGF expression using *in situ* hybridization. They also used molecular histochemistry to compare pancreatic remodelling at birth and postnatal days 14 and 21 in offspring of LP and C mothers. They used bromo-deoxyuridine (BrdU) to stain for cells actively synthesizing DNA and TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling) method to stain for apoptosing cells. To further examine which cell cycle events were occurring *in situ,* they performed immunohistochemistry for: NEK2 (NIMA related kinase 2) an indicator of cells in G2 and mitosis; cyclin D1 which is a marker of G1; and for proliferating cell nuclear antigen (PCNA) which is present in G1 as well as S and G2 phase.

Petrik et al (1999) reported that, IGF-II expression declined postnatally and by postnatal day 14 was barely detectable in both groups of pups. In LP offspring, IGF-II expression was reduced compared to C pups at all ages. Also, there was a reduction in the number of islets staining for immunoreactive IGF-II in LP vs. C pups. They found that IGF-II was predominantly expressed in the islet and was less abundant in pancreata of LP offspring at postnatal day 2. By postnatal day 21, IGF-I was located in the acinar cells, but its expression did not vary between the diet groups.

Petrik et al (1999) reported a significantly lower percent of islet cells labelled with BrdU in LP pups compared to C pups at all three ages. Unlike BrdU, the percentage of islets that were immunopositive for PCNA was greater in the LP group compared to C group at each age. They found that in LP offspring, a greater percentage of ß-cells were in the G1 phase or the gap between mitosis and DNA synthesis, whereas a fewer

percentage of ß-cells were in S phase or G2/M or DNA synthesis. They concluded that this result could explain why LP offspring have fewer ß-cells. However, even though a 50% reduction in ß-cells has been observed in islets from LP offspring, it is has been suggested that an 80-90% loss is required to produce diabetes (Hales & Barker, 1992).

While Snoek et al (1990) and Petrik et al (1999) have shown that a LP diet fed to pregnant rat dams leads to changes in foetal pancreatic structure; there are several important caveats. For example, do these alterations lead to diabetes in adult offspring of mothers fed an LP diet; and if so, at what age? Similarly, does feeding the mother a LP diet during lactation pronounce the effects observed on pancreas structure? Does pancreatic structure recover if the pup is fed a protein sufficient diet from birth?

To answer such questions, Dahri et al (1991) studied three groups of rats: a control (C) group of offspring from rat dams that were fed a control diet during pregnancy and lactation and were weaned onto a control diet; a low protein (LP) group consisting of offspring of mothers fed a LP diet during pregnancy and lactation and were weaned onto a LP diet; and a recuperated (R) group of offspring from rat dams fed an LP diet during gestation but were fostered after birth to dams fed a protein sufficient (control) diet and were weaned onto the C diet. They measured insulin secretion and islet insulin content in samples of 10 islets isolated from foetal rat pancreata (21.5 days gestation) after incubation (30 and 120 minutes) with various secretagogues: glucose (0, 2.8, 5.6 and 16.7 mM), leucine, arginine and theophyilline. After 15 hours of fasting they conducted oral glucose tolerance tests (OGTT) in rats at 28, 56 and 84 days of age.

Dahri et al (1991) found that insulin secretion from islets in response to the amino acids and theophylline was significantly lower in foetal islets from LP mothers after 30

and 120 minutes of incubation. Rats from LP mothers ate less food than R or C rats and gained less weight than R or C rats after weaning. Although fasting blood sugar increased with age in all 3 groups of offspring, it was not significantly different in the three groups. At 70 days of age, rats in the LP group exhibited impaired glucose tolerance since their blood sugar reached a peak of 9mM glucose (6.9 mM control) at 30 minutes and by 120 minutes it was 6.5 mM and had not returned to basal levels (5 mM) unlike in the C or R rats. Also, plasma insulin concentrations in LP rats at 30 minutes were almost half the concentration observed in C and R rats. Desai et al (1995) used this same design but studied glucose tolerance in later adulthood (15 months). They found that by later adulthood rats from the LP group were less glucose tolerant than the controls (Hales et al, 1996).

Hales & Barker (1992) hypothesized that during periods of nutrient deprivation, the foetus diverts nutrients towards essential organs such as the brain and heart, at the expense of muscles and organs like the liver and pancreas. Initial studies demonstrated that the foetal endocrine pancreas was altered by maternal protein restriction. However, studies have indicated that it is possible that other organs involved in glucose metabolism may be 'programmed' *in utero* and contribute to the development of T2D. For example, Ozanne and colleagues conducted three studies to investigate the effects on glucose metabolism: 1) in the liver (Ozanne et al, 1996a); 2) in skeletal muscle (Ozanne et al, 1996b); and 3) in adipocytes in male offspring of rat dams fed the LP diet during pregnancy and lactation but weaned on to rat chow at three weeks of age (Ozanne et al, 1997).

At three months, male LP offspring had a lower body weight than control animals and had significant increases in glucose transport that were accompanied by alterations insulin signaling pathway molecules (increased IRS-1 and p85-PI3kinase, but decreased p110-PI3kinase) (Ozanne et al, 1997). There was evidence of several adaptations in livers from LP rats: hepatic glucose output (HGO) was significantly greater compared to control rats and there was a three-fold increase in GLUT-2 transporter protein in hepatic plasma membranes from LP animals (Ozanne et al, 1996a). Liver cell membranes from LP rats had 3 times as many insulin receptors vs. those from C rats; whereas, there were significantly fewer glucagon receptors in LP hepatic cell membranes. A reduction in insulin inhibition of HGO at 3 months of age in rats (which was still observed at 11 months) suggests that maternal protein restriction also leads to permanent alterations in the liver that could lead to increased risk of T2D (DeFronzo et al, 1992). To address the effects of maternal protein restriction on insulin sensitivity it is important to study aspects of glucose homeostasis in peripheral tissues.

Ozanne et al (1996b) also examined the relationships between insulin stimulated glucose transport in skeletal muscle (tibialis anterior) at high (16 nM) and low (300 pM) concentrations of insulin following 10 minute and one-hour incubation (Krebs-Ringer buffer, pyruvate, mannitol and BSA) and GLUT-4 glucose transporter subcellular distribution and insulin receptor number in male LP rats at 3 months. They reported that pre-incubated (10 minutes) muscle strips from LP animals had significantly higher basal glucose transport rate ($nmol \cdot min^{-1} \cdot mg$ wet skeletal muscle $weight^{-1}$) compared to muscle from LP pups. After incubating with higher insulin concentrations (16 nM) they found

glucose transport increased sixfold in muscle from C pups compared to a 1.5-fold increase in LP rats.

Following the longer pre-incubation period (1 hr), they found that the basal rates of glucose transport into skeletal muscle were not significantly different. However, there was greater glucose transport into LP muscle at the lower concentration of insulin (300 pM) and no difference was detected in the higher concentration of insulin (16nM). Under basal conditions, muscle from LP rats had significantly more GLUT-4 protein in the plasma membrane compared to C rats and muscle from LP rats had twice as many insulin receptors.

Ozanne et al (1997) used the same model described above to determine whether there is a deterioration of glucose transport into the adipocyte associated with insulin resistance. They isolated adipocytes from LP rats and C rats and compared whether insulin signalling pathways were different. They found that adipocytes from LP offspring had greater glucose uptake under basal conditions and in the presence of insulin (80 nM). In both conditions there was increased insulin stimulated insulin receptor substrate (IRS)-1 associated phosphatidylinositol 3 kinase (PI 3-kinase) activity in adipocytes from LP rats compared with control. Also, using Western blot analysis, there were three times as many insulin receptors (per milligram of plasma membrane protein) in adipocytes prepared from LP animals (Ozanne et al, 1997).

Observations from these three studies (Ozanne et al, 1996a; Ozanne et al, 1996b; Ozanne et al, 1997) suggest that peripheral tissues (adipocytes and muscle) in LP offspring seem to be more sensitive to insulin. Since there were no differences observed in GLUT-4 expression or distribution, Ozanne et al (1997) concluded the increased

insulin sensitivity is likely due to alterations in insulin signalling pathways. The consistently greater numbers of insulin receptors expressed in hepatocytes, muscle and adipocytes may contribute to the insulin responsiveness seen observed in LP offspring.

Wilson & Hughes (1998) hypothesized that a nutritional stimulus in adulthood (e.g. high fat or high carbohydrate diet) is necessary to expose underlying abnormalities in ß-cell function that lead to impaired glucose tolerance. They used a modified LP model and fed rat dams either a control (18% protein) or a low (5% protein) diet during pregnancy and lactation. Offspring were weaned on to a control diet at 21-28 days until 10 weeks when some animals were allowed free access to a 35% sucrose solution. They found that poor nutrition during the foetal-neonatal period impaired glucose stimulated insulin release in adult offspring by 49-55%. Therefore, feeding dams a LP diet during pregnancy is strongly associated with alterations in the development of the foetal endocrine pancreas and tissues involved in glucose homeostasis, such as the liver, muscle and adipocytes.

Bertin et al (1999) developed a slightly different model to examine the long-term effects of foetal malnutrition on pancreas development during the last week of gestation. Their design also attempted to separate the effects of protein malnutrition from energy malnutrition. Therefore, they fed pregnant rats standard rat chow *ad libitum* during the first two weeks of gestation and then assigned these dams to one of four experimental conditions during the last week of gestation: 1) energy restriction to 50% of their standard pregnancy intake (CER); 2) energy restriction to 50% pregnancy intake using a low protein diet (PER); 3) a low protein diet pair fed to energy intakes of control rats (PR); or 4) a control diet (C). Pups were fostered to rat dams and fed a control diet during

pregnancy and lactation. They were then weaned onto a standard diet at postnatal day 28 until 8 weeks of age. Only female offspring were included.

Bertin et al (1999) found that maternal weight gain was affected by diet. For example, at 21d gestation CER and PR rat dams gained less weight than C rats and the PER group had lost weight after one week of nutrient restriction. However, only offspring born to mothers fed restricted protein diets (PR and PER) had lower birth weights compared to C pups. At 8 weeks of age there were no significant differences in body weight, glucose intolerance, glucose utilization or glucose production in the offspring (Bertin et al, 1999). However, pancreatic insulin content and β-cell mass were significantly lower in the PR group. This showed that the developing pancreas was more sensitive to protein restriction than energy restriction alone.

Berney et al (1997) modified the LP model to determine if the pancreas was more sensitive to protein restriction during gestation or suckling. Using C and LP pups, they added two other groups: a postnatal LP (postLP) group of offspring from mothers fed a control diet during pregnancy and a LP diet during lactation; and a prenatal low protein (preLP) group of offspring from mothers fed a LP diet during pregnancy and a control diet during lactation. All rats were investigated at the end of the suckling period (21 days of age).

Body weight varied among pups that were subjected to maternal deprivation at different stages of development. Although the preLP group weighed significantly less than C pups, there was a greater difference observed between the LP and postLP groups compared to C pups. Pancreata from offspring of LP and postLP mothers weighed significantly less compared to the control group and there was no difference in pancreatic

weight between the preLP and C group. Total β-cell mass and percentage beta-cells were reduced in all three treatment groups compared to control rats. However, when total islet cell mass, β-cell mass and α-cell mass are compared, controlling for body weight, there were fewer differences between the groups.

Berney et al (1997) showed that feeding pregnant dams a diet low in protein, initiated both at pregnancy and at birth, could lead to alterations in the endocrine pancreas that could predispose to T2D. Since they did not investigate whether these adaptations persisted through to adulthood, it is unclear whether ß-cell recovery follows weight or remains programmed. An interesting component of the animal research shows that despite optimal nutrition *in utero* the developing pancreas is still susceptible to permanent alterations as a result of neonatal diet.

2.2 Metabolic Imprinting in the Postnatal Period

2.2.1 Association Between Postnatal Nutrition and T2D Risk in Humans

While epidemiological studies to date have focused on the foetal period as a critical period for altering metabolic risk, there may be other critical periods of development. There are very few studies examining the relationship between nutrition during infancy, childhood or adolescence and later chronic diseases such as CVD or T2D. For example, Fernandes et al (2003) measured arterial blood pressure (AP) of 60 children (10-16 years of age) living in shantytowns in Brazil. They selected children with some degree of nutritional deficiency (based on anthropometrics such as skin fold thickness or short stature) and classified them according to Tanner & Whitehouse (1962) for pubertal stage.

They found that based on age, children in their study had a slight delay (one degree) in pubertal staging. They reported that 19% of boys and 23% of girls had diastolic hypertension and 33% of boys and 27% of girls were at risk for hypertension. For systolic AP, 12% of girls were above the 90th percentile and therefore were at risk for systolic hypertension.. In addition, 6% of subjects exhibited simultaneous systolic and diastolic arterial hypertension.

This study shows that malnutrition up to the time of adolescence may predispose individuals for increased risk of hypertension in adulthood. However, since these authors did not control for birth size there could be confounding between adolescent malnutrition with malnutrition during pre or postnatal periods. Therefore, to understand the influence of post-natal nutrition and risk for T2D animal models are required.

2.2.2 Effects of Dietary Carbohydrate During the Suckling-Weaning Period in the Rat

During suckling (0-14 days of age), the rat pup depends on mother's milk as its sole source of nutrition. At 15 days of age, a transition occurs whereby animals begin to wean from a diet that is relatively high in fat and low in carbohydrate to one that is higher in carbohydrate and lower in fat. This nutritional transition may be important for the developing pancreas since it may be 'imprinted' and undergo important metabolic adaptations during this period. Models used to explore the effects of post-natal nutrition have modified diet either during suckling or immediately post-weaning.

Literature examining the effect of diet during early post-natal life can be generally divided into two groups: 1) those changing diet prior to weaning, or 2) those changing diet in the early post-weaning period. The two models that have been used to study the

influence of suckling diet and later life are litter size modification studies (McCance, 1962) and studies using an artificial rearing technique which will be discussed in the next section. Studies that examine the early post-weaning diet use a low protein model (Reis et al, 1997), a protein and calorie restriction (Swenne et al, 1988), or wean animals on to a high fat or high carbohydrate diet introduced either prematurely at 15 days (Vernon & Walker, 1968) or at 17-21 days of age (Issad et al, 1988).

McCance (1962) studied the effects of litter size on growth rates during suckling. In his study he raised rats from two different litters born on the same day and reared them in litter sizes of three and compared their growth rates to those reared in litters of 15-18 pups. At three weeks of age the rats suckled in litters of three weighed two to four times more than those suckled in the larger litters. Pups in the large litters continued to grow more slowly and the difference in body weight between those in raised in the small versus large litters increased with age. Those raised in the large litters never attained the length or weight of those in the small litter despite unlimited access to food.

Further study showed that if the nutritional restriction occurred after a certain 'window' then the short-term reduction in body weight was not permanent. For example, rats that were nutritionally deprived at 9 to 12 weeks of age, re-gained weight to match littermates whose intake had never been restricted. This study demonstrates that over-nutrition during suckling may cause permanent alterations in growth and suggests that long-term alterations in growth could have origins in early post-natal life.

Several investigators have looked at the effects of introducing a solid diet prior to 'natural weaning'. Vernon & Walker (1968) studied the effects of several dietary modifications on liver enzyme activities in the rat during the weaning period. These

authors previously showed that rats could be weaned at 17 days of age, although it is a common practice to wait until 21 days. They conducted three separate experiments to examine the effect of timing (early vs. late weaning) and composition of weaning diet. Group One was given either a high carbohydrate (HC) or high fat (HF) liquid diet and was pre-weaned at 12-15 or at 15-18 days of age. Group Two was weaned either at 17-18 or 21 days of age onto a HC (30% glucose; 70% chow) or standard rat chow. Group Three was allowed to wean naturally at 21-24 days onto either a high protein or high fat diet in the presence of a lactating mother.

Pups that were fed a HC diet at 12 days had greater hepatic pyruvate kinase activity at an earlier age than those weaned after 17 days. Similarly, rats that were weaned at 18 or 21 days had higher hepatic pyruvate kinase activities than natually-weaned animals. Those fed the HC diet had even higher enzyme activity levels. If animals were allowed to wean naturally, the normal increase in pyruvate kinase activity was lower in pups fed a HP diet and totally inhibited by a HF diet. ATP citrate lyase and malic enzyme (ME) were also significantly inhibited by the HF diet, whereas those fed the HC diet had higher ME activity levels than the control pups. Vernon & Walker (1968) found that weaning prematurely onto a HC diet will very rapidly induce high glucokinase activities once the liver is able to synthesize these enzymes.

Angel & Back (1981) conducted a similar study to investigate the immediate and long term effects of premature weaning on enzymes involved in lipogenesis (hepatic glucose-6-phosphate dehydrogenase and ME) and whether incorporating a low level of sucrose had an additive effect. A preliminary experiment was conducted whereby 16 day old rats were weaned onto a diet with various amounts of sucrose (0, 10, 20 or 30% kcal)

and hepatic enzyme activity levels were measured at 31 days of age. They determined that 20% of energy from sucrose was sufficient to observe increased hepatic glucose-6-phosphate dehydrogenase (G6PD) and ME activities. The second experiment involved three separate diet groups: one in which rats were weaned at 17 days to the control diet (PWC); one in which sucrose provided 20% kcal (PWS); and one in which pups were left with dams until 30 days of age (NWC). All groups were maintained on control diet from 31 days of age to 122 days.

The authors found that livers from PWC pups had increased G6PD activity by postnatal day 25 and livers from pups in the PWS group had increased enzyme activities by day 19. All pups, including the NWC pups, had considerable G6PD activities at day 18 although ME was barely detectable. Premature weaning caused an early increase in ME activity that could be observed from day 19 and after 14 days of feeding ME activity was two times higher in the sucrose fed group. However, levels of liver or carcass fatty acid synthesis were not different between the NWC and PWC group as determined by incorporation of ^{3}H from tritiated water. Sucrose feeding resulted in approximately twice the fatty acid synthesis in the liver and in the carcass. While sucrose feeding before weaning was associated with higher lipogenic enzyme activities at 30 days of age, there was no difference detected in adult rats. The results from this study suggest that dietary carbohydrate in the pre-weaning period could be a 'signal' for increased lipogenic enzyme activity.

Issad et al (1988) studied rats that were maintained on a high fat diet from days 14-30 of age or weaned onto a high carbohydrate diet. They found that at 30 days of age, blood glucose concentrations were significantly higher in rats weaned on to the HF diet

than both suckling pups and pups weaned on to the HC diet. Plasma insulin concentrations were lower in the suckling and HF-weaned rats than in the HC-weaned rats; whereas plasma glucagon concentrations were higher in the suckling and HF-weaned rats than in the HC-weaned rats. Suckling rats had higher plasma insulin concentrations during the euglycemic-hyperinsulinemic clamp studies despite similar insulin infusion rates, suggesting a lower rate of insulin clearance.

Issad et al (1988) also reported much lower rates of glucose utilization and metabolic clearance of glucose in suckling pups compared to weaned pups. While glucose utilization rates were similar between the HF and HC rats after weaning, HF fed pups showed a lower metabolic clearance rate of glucose than the HC pups. Similarly, endogenous glucose production was suppressed by more than 90% in the HC pups but was only inhibited by 40% in HF pups. This study shows that it is possible to alter glucose production and utilization by changing diet during this period. It is not clear from this study whether these effects last into adulthood.

3. Artificial Rearing Model Allows Nutrient Alteration in the Suckling Period

Investigating the effect of the suckling diet on metabolism is challenging since the infant relies on its mother for nourishment. As discussed previously, McCance et al (1962) showed that modifying litter size could lead to long-term alterations in growth. Other studies, like those described by Ozanne et al, restrict dietary protein during pregnancy and lactation and have shown long term affects on glucose metabolism. Since these models modify the pups' diets indirectly, they do not allow for precise control over the nutritional intake during the suckling period. A way to directly

manipulate nutrient intake in rat pups is known as artificial rearing. Using this method it is possible to investigate the effects of suckling diet in an animal model using an artificial milk substitute.

This technique has been used for more than 50 years and initially used small animals such as rats, mice and rabbits raised in germ-free conditions. Early research using AR involved either hand-feeding a rat milk substitute by offering a rubber nipple at hourly intervals (Pleasants, 1959) or force-feeding a milk formula via a syringe needle fitted with a bulbous tip (gavage) (Gustafsson, 1946; Miller & Dymsza, 1963; Czajka-Narins & Hirsch, 1974). Many problems were associated with early AR experiments; for example, hand-feeding rat pups required careful attention to the amount fed (Gustafsson, 1946; Pleasants, 1959). They were also labour intensive and associated with high rates of injury since the procedure involved repeatedly inserting a syringe down the pup's oesophagus (Gustafsson, 1946; Miller & Dymsza, 1963).

The first surgical technique to artificially feed neonatal rats by continuous gastric infusion was described by Messer et al (1969). Messer's technique is currently being used based on a method described by Hall (1975) that uses a less traumatic procedure to implant an intragastric cannula. The two major components of this technique are a miniaturized intragastric tube and an incubator housing arrangement. Since the pups were housed in Styrofoam® cups in a temperature controlled water bath, this technique has been coined the 'pup in a cup' method. Hall (1975) applied this method to feeding pups as young as two days of age. Using this model, pups are fed a pre-determined amount of formula per day (approximately 2.092 kJ/g body weight) and is adjusted to 12-24 feedings per day (on a one or two-hour cycle). Success varies from 60%-90% and the

greatest losses occur from trauma during surgery and from bloating caused by gastrointestinal distention (Patel & Hiremagalur, 1992). Losses can be minimized by the addition of sodium deoxycholate (0.01 g/100 g formula; Diaz et al, 1982). Pups that we reared using this method were similar in body weight to mother-fed control pups (Hall, 1975; Patel & Hiremagalur, 1992).

There are several advantages to using rats in the AR model. The large number of pups born to rat dams allows greater experimental control since pups can be randomly assigned to many experimental groups. Also, since rats are small animals they are relatively inexpensive to house and feed and the design and assembly of the experimental apparatus are comparatively easier (Patel & Vadlamudi, 1994). In addition, the rat has been studied extensively in nutrition research its nutritional requirements are well-defined.

The AR technique has since been applied to several areas of investigation, including: study of the nutritional and hormonal influences on intestinal cell growth and enzyme expression (Yeh et al, 1986); the influence of formula feeding on brain and gut development (Kanno et al, 1996); the effects of n-3 essential fatty acid deficient formula (Ward et al, 1996); the effects of milk-borne insulin (Kinouchi et al, 2000) and other polyamines (Pollack et al, 1992); the lasting effects of maternal deprivation on behaviour (Smart et al, 1989); the effects of overfeeding to accelerate weight gain (West et al,1982); the effects of ethanol exposure (Ward & West, 1992); and the effects of a high carbohydrate diet during suckling (Vadlamudi et al, 1993).

3.1 Feeding an HC Formula During Suckling Causes Permanent Alterations in Pancreatic Function

Results from previous studies show that the suckling-weaning transition in rats is associated with several metabolic adaptations. However, the transition from foetal to neonatal diet at birth may be another important period. *In utero* the developing foetus relies on intravenous glucose for energy and at birth receives a high fat liquid diet from mother's milk. During the rat's last 4-5 days of gestation, known as the late foetal period, there is induction of enzyme activities associated with glycogen synthesis and degradation (Patel & Hiremagalur, 1992). Within the first few hours after birth, the rat develops gluconeogenic capacity and this coincides with the appearance of cytosolic phosphoenolpyruvate carboxykinase. Similarly, the transition to a high carbohydrate diet at weaning is associated with the emergence of hepatic glucokinase. Overall, the high fat content of milk is associated with a suppression of hepatic lipogenesis while there are several enzymes which appear during the later suckling period, which is associated with the transition to a high carbohydrate diet.

Haney et al (1986) adapted the AR model to feed a HC formula (56% carbohydrate, 20% fat, 24% protein) from one day of age until weaning and used only a mother fed (MF) control. They reported that in mother- reared rats, plasma insulin concentrations decline shortly after birth, whereas in HC rats there was a threefold higher level than MF pups. There was also a three-fold higher rate of lipid synthesis measured using ^{3}H incorporation from $^{3}H_2O$ into lipids and increased activity of G6PD and fatty acid synthase activities in HC fed rats compared with MF controls. This suggests that a HF diet during suckling influences plasma insulin to suppress hepatic lipid synthesis. Before the AR technique had been developed, researchers had concluded that the

neonatal liver was unable to synthesize ME or glucokinase (Vernon & Walker, 1968). However, Patel & Hiremagalur (1992) showed that rats fed a HC diet during the first few days of life induced premature hepatic malic enzyme (ME) activity. This suggests that the liver is able to adapt to dietary and hormonal changes early in post-natal life.

According to Henning (1981) expression of enzymes in the late suckling period occurs concomitantly with changes in several hormones. For example, the principal carbohydrate in milk is lactose during which time the brush border enzyme lactase is high at birth until 2 weeks. The sucrase enzyme is virtually absent at birth and markedly increased after 3 weeks of age. The appearance of intestinal sucrase activity during postnatal week 3 is preceded by changes in total serum concentration of corticosterone. In early studies using the AR technique, Yeh et al (1986) showed that 12 day old rats that had undergone intragastric cannula implantation, either fed by mothers or reared artificially, had a transient surge in corticosterone activities 48 hours following surgery. If the same pups underwent adrenalectomy, then the induction of sucrase activity did not occur. However, if adrenalectomized AR rats were given corticosterone, then precocious induction of sucrase activity was restored. Therefore, using this model it is impossible to determine whether the differences in hepatic enzyme activities observed in the HC pups were due to the early introduction of carbohydrate or the AR technique. This limitation can be avoided by the inclusion of an artificially reared control, fed a HF similar in composition to the suckling control or mother-fed pups.

Patel & Hiremagalur (1992) further adapted this model to feed rats from 4 days of age until weaning and included another group of rats fed a high fat (HF) diet (8% carbohydrate, 68% fat, 24% protein) to control for differences associated with the AR

technique. Vadlamudi et al (1993) studied both the long and short-term effects of nutritional modification during the suckling period. For the short-term studies, they measured pancreatic hormone concentrations at day 12 in pups fed, HF, HC or MF diets. For the long-term studies, they weaned HC, and MF pups onto chow at 24 days and switched them to a high sucrose diet (52% sucrose, 15% starch, 21% protein, 15% fat) at day 64 until day 100. Another group of HC and MF pups were weaned onto regular lab chow at day 24 and studied until 280 days. Oral glucose tolerance tests (OGTTs) were conducted on day 64 before the switch to the high sucrose diet and again on day 78.

Vadlamudi et al (1993) did not observe any significant difference in body weight until day 54 when HC rats weighed on average 349 g (MF 316g; HF 314g). By day 100 HC rats weighed approximately 100 grams more than the other rats (HC 710 g; MF 610g; HF 603g). On days 12, 54 and 100 HC pups had higher plasma concentrations of insulin compared to MF or HF pups. While there were no differences in plasma concentrations of glucose or glucagon detected among the three groups at 12 days of age, by 280 days both plasma glucose (HC 6.26 mmol/L; MF 5.16 mmol/L) and plasma insulin concentrations (HC 821 pmol/L; MF 390 pmol/L) were higher in HC rats compared to MF rats.

At 64 days of age there was no difference in first phase insulin response to an oral glucose challenge, but after 60 minutes HC rats had higher plasma concentrations of insulin and glucose compared to MF control rats. On day 78, two weeks after sucrose feeding was initiated, plasma glucose levels were higher in the HC group at all time points compared to MF or HF rats. Whereas, insulin secretion in response to the glucose challenge after 10 minutes was impaired in HC rats, after 30-120 minutes the insulin

response was more pronounced in HC rats compared to MF or HF rats. Similar impairments in insulin secretion were observed in HC rats that did not undergo the high-sucrose challenge. Differences were observed in the morphometry of HC pancreatic islets on day 12 and there were more insulin-positive stained cells in HC rats compared to MF or HF controls, which was also observed at day 100. While mean islet size was significantly greater on day 12, on day 100 there was no significant difference among the three diet groups.

Studies by this group have since focused exclusively on the mechanisms underlying the persistence of hyperinsulinemia associated with a HC diet during suckling. Aalinkeel et al (1999) studied the insulin secretory pattern and glucose transport in islets of HC rats at 12 days of age. They found that despite six-fold higher plasma insulin concentration in HC pups, there were decreased concentrations of plasma-free fatty acids (FFA) and triglycerides (TG) compared with MF controls. Glucose stimulated insulin secretion studies showed that MF islets did not secrete any measurable insulin at 1 mM glucose or at 2.8 mM at 10 minutes, and very small amounts (0.23 fmol/30 islets) at 60 minutes. Calcium channel inhibitors suppressed *in vitro* glucose stimulated insulin secretion by ~40% in HC islets, whereas insulin secretion from MF islets was undetectable. Under potassium channel depolarization or treatment with 25 mM potassium chloride or 100 μM glibeclamide, MF islets secreted significantly more insulin but did not have an effect on insulin secretion in HC islets. GLUT-2 protein content was measured using Western blot analysis and was approximately 70% higher in HC islets compared to MF. Similarly, there was a 40% increase in glucokinase activity. However,

this leftward shift in insulin secretory response to glucose does not completely account for the six-fold increase in circulating insulin at 12 days of age.

Srinivasan et al (2000) conducted further studies to determine which molecular events may underlie the development of hyperinsulinemia in HC rats. Incretin hormones (e.g. glucagon-like peptide, or GLP) in the gastrointestinal tract are responsible for a greater insulin secretory response to ingested glucose than intravenous glucose (Kieffer & Habener, 1999). Srinivasan et al (2000) observed a nearly threefold higher plasma level of GLP-1 compared to MF rats, which corresponded with a nearly threefold higher increase in mRNA levels of GLP-1 receptor from 12 day old HC islets, which was measured using PCR. Similarly, activities of protein kinase A, protein kinase C and calcium calmodulin (CaM) kinase II, which modulate insulin secretion by β-cells, were all increased in HC islets compared to islets from MF rats. Further investigations showed that a ~10 fold higher norepinephrine concentration, which is a physiological antagonist of insulin, was required to achieve the same degree of insulin inhibition in HC islets compared to MF islets. Later, a cDNA array was conducted to compare gene expression in HC and MF islets at 12 and 100 days of age (Song et al, 2001). Among the genes that were upregulated in HC islets, only a few were known to have an effect on insulin secretion. Song et al (2001) reported that, "global changes in gene expression contribute to the hyperinsulinemic state in the HC rat." More specifically, significant upregulation was observed for G-protein related factors, sodium channels, cholecystokinin receptor gene, acetyl-CoA carboxylase and cytochrome oxidase II.

Since previous study using this model has shown that gene expression is altered in HC rats, Laychock et al (1995) and Vadlamudi et al (1995) examined the persistence of

hyperinsulinemia in further generations of HC rats. On day 60, male and female rats from the same dietary group (6 MF, 4 HF, and 4 HC) were bred. Their natural mothers nursed the resulting progeny until postnatal day 24 when they were weaned onto rat chow. Vadlamudi et al (1995) maintained the rats until investigation on day 100, whereas Laychock et al (1995) conducted islet isolation and secretion experiments at 250 days of age. Milk macronutrient composition on day 14 was measured by Laychock et al (1995) and they found no significant differences.

3.2 Feeding an HC Formula During Suckling has Effects on Pancreatic Function in the Second Generation

Vadlamudi et al (1995) reported that plasma insulin concentrations were higher in HC mothers before and during pregnancy. However, there was no significant difference detected during lactation in HC mothers compared to MF and HF mothers or difference in birth weight or growth rate among the offspring from MF, HC or HF mothers. However, plasma insulin concentrations were higher in both second generation HC male and female offspring compared to HF and MF controls. Hyperinsulinemia in the progeny of HC parents was observed at 45, 65 and 100 days when it was approximately 113% greater than MF progeny and 74% greater than HF progeny. Fatty acid synthase and G6PD activities were also significantly increased in HC offspring compared to HF and MF offspring. When islet insulin response to glucose *in vitro* was compared among offspring of the three diet groups at 100 days, fasting plasma insulin concentrations and basal insulin release were higher in second generation HC rats. However, glucose stimulated insulin release from second generation MF rats between 10 and 60 minutes was higher compared to HF or HC offspring.

These studies show that altering the nutritional composition of the suckling diet can contribute to the 'programming' of insulin secretion in the rat. The suckling period overlaps with a critical period of development in the endocrine pancreas and has been shown to undergo significant adaptations to a HC diet fed during the first three weeks of postnatal life. It is associated with the immediate onset of hyperinsulinemia, obesity later in life, and extensive biochemical and molecular changes that appear to be a phenotype passed on to their progeny. The HC model uses sucrose as the carbohydrate source, which contains fructose (FR). Under natural suckling conditions, lactose is the sole carbohydrate, and the diet gradually includes increased intake of other carbohydrate sources. Since the HC model, developed by Patel et al, involves the addition of sucrose to achieve the high carbohydrate content it is unclear whether the results are due to low fat intake, premature fructose intake or both.

4. Fructose

4.1 Increased Fructose Consumption in Humans

Fructose is a naturally occurring monosaccharide found in vegetables and fruit and is one half of the sucrose dissaccharide with glucose. Fructose is a significant and increasing source of dietary carbohydrate in Western diets. From 1970 to 1997 the estimated daily per capita fructose consumption (calculated using disappearance data) increased approximately 26% from 64 g/day to 81 g/day (Elliott et al, 2002).

Harnack et al (1999) showed that school-aged children who were consumers of soft drinks (9 oz soda/day) were consuming ~200 kcal more per day than non-soft drink consumers and that soft drinks were replacing other dietary beverages such as milk and

fruit juice. Similar findings have been reported in Spain (Rodriguez-Artalejo et al, 2003) and Australia (Somerset, 2003). Further investigation into the intake of refined sugar intake of Australian children revealed an approximate mean daily intake of 27 g - 80 g per day in girls and boys (aged 2-18 years), or approximately 7-15% of their daily energy intake. Of the 10 highest sources of refined sugar intake, sweetened beverages (especially cola) were the most common.

Dietary FR consumption at an early age in human is associated with the increased consumption of sweet beverages, along with the increased consumption of breakfast cereals, baked goods, condiments and prepared desserts made with high fructose corn syrup (HFCS) (Elliott et al, 2002). According to Park & Yetley (1993), HFCS consumption began to increase in the 1970's and by 1985 accounted for approximately 35% of the total amount of sweetener use in the food supply. Most of the HFCS used in beverages contains ~55% FR. Using data collected from 1977-1978, food consumption surveys by Park & Yetley (1993) estimated that young males (15-18 years), were the largest consumers of FR with daily intakes of ~100 g/d.This figure could be grossly underestimated in view of increased beverage consumption over the past three decades. Elliott et al (2002) calculated that two x 355 mL of soda could supply approximately 50 g of FR, or >10% of the energy requirement for an average weight woman. Therefore, it is important to examine whether increased FR consumption is associated with increased rates of T2D or insulin resistance.

4.2 Fructose Absorption

According to David et al (1995), approximately 40% of the energy intake by human infants is through carbohydrates. Intestinal glucose transport appears prenatally at the 24th week of gestation in humans and just before birth in rats (Lebenthal et al, 1983). However, the development of fructose transport occurs at weaning and in the absence of dietary fructose in the rat (Buddington & Diamond, 1989). Since dietary fructose only appears at weaning, David et al (1995) studied the dietary induction and regulation of intestinal FR and glucose transport.

David et al (1995) studied the effects of early weaning using a high glucose (HG), high fructose (HFR) or mother fed controls from 16 days of age. Studies during late weaning involved feeding rats from 22 days, using mother-fed control, HG, HFR, high sucrose (HS) or no carbohydrate (NC) groups. Similar studies were conducted in older rats that were fed either normal rat chow, HG, HFR, HS or NC diets from 35 or 60 days of age. All rats were fed their respective diets for 6-7 days. At the end of the feeding trial, glucose and fructose uptake into the small intestinal mucosa was measured by using everted sleeves technique described by Karasov & Diamond (1983).

In 16-22 day old pups, dietary FR intake stimulated FR absorption by 1.6 times in the proximal and 1.4 times in the middle intestine. It did not affect absorption in the distal intestine. Similar observations were made in the small intestine (SI), where dietary FR enhanced FR absorption by 1.3-1.5 times in the proximal and middle SI. FR absorption also depended on diet and intestinal position in rats at 21-28 days of age. Similar observations were made among HFR and HS rats at 42 and 67 days of age. Therefore, FR transport can be induced before weaning in the rat and can is a product of sucrose

hydrolysis or in the diet as an additive as free FR. The increased levels of FR absorption in older HFR and HS rats, compared to mother-fed controls that were weaned to normal rat chow and still exposed to dietary FR, suggests there may be a threshold level of FR required to induce transport. However, it is unclear whether FR transport can be induced during suckling. Our lab is presently investigating this question.

Sugar absorption from the lumen of the small intestine by mature enterocytes lining the villus (Figure 3) requires specific transport systems for the three major dietary hexoses (glucose, galactose and fructose). Active transport involves the use of sodium dependent glucose transporters (SGLTs) in the brush border membrane (Diez-Sampedro et al, 2001; Ferraris, 2001). Evidence for their role in dietary glucose and galactose absorption comes from studies in humans with glucose and galactose malabsorption who lacked functional SGLT-1 in their intestinal brush border (Ferraris, 2001). They describe three members of SGLT 'family': SGLT-1 and SGLT-2 that are involved in sugar transport or as a glucose sensor (SGLT-3) (Scheepers et al, 2004). The active transport of glucose or galactose involves the consumption of adenosine triphosphate (ATP) when sodium (Na+) is pumped out of the cell into the lumen of the small intestine or into the blood. This gradient results in the co-transport of one molecule each of sodium and glucose. Next, the monosaccharide is transported across the cell into the bloodstream via GLUT2. Similar to glucose, galactose uses the SGLT-1 cotransporter and GLUT 2.

Other transporters that exist in the small intestine transport independently of sodium and ATP. They are facilitated by the concentration gradient and by energy potential that exists across the cell membrane. The facilitative fructose transporter is GLUT-5 which transports absorbed fructose across the brush border membrane (Thiesen

et al, 2000; Ferraris, 2001) out of the enterocyte and across the basolateral membrane using GLUT2.

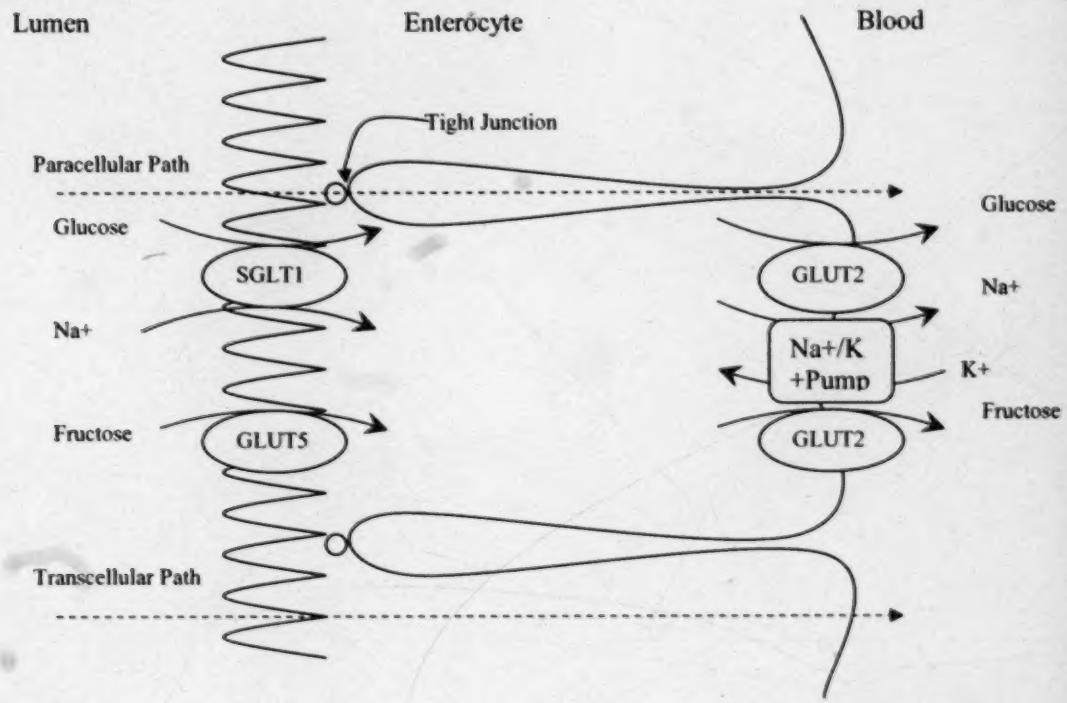


Figure 3. Absorption of dietary FR (Adapted from Thiesen et al, 2000 and Ferraris & Diamond, 1997).

4.3 Fructose Metabolism

The small intestine in some species, specifically the golden hamster, guinea pig and dog, is able to convert some absorbed fructose into glucose (Mayes, 1993). Therefore, the rat is a good model to study fructose metabolism since the rat metabolizes FR in a way similar to humans. As such, absorbed fructose is delivered to the liver via the portal vein; therefore, all dietary fructose goes through the liver initially (Mayes, 1993). According to Mayes (1993), a limitation of studies using fructose is that they use unphysiological levels of fructose and create misleading observations. In humans, the maximum plasma fructose concentration reached was 2.2 mmol/L, following meals high in fructose or sucrose (Holdsworth & Dawson, 1965) compared to 1.1-2.2 mmol/L in rats (Topping & Mayes, 1971).

In the liver, fructose is rapidly phosphorylated (Figure 4) by adenosine triphosphate (ATP) to form fructose-1-phosphate. The reaction is catalyzed by the enzyme fructokinase. Since fructose is the only significant dietary ketohexose this enzyme is specific only for fructose. Fructose-1-phosphate is split by aldolase B into glyceraldehydes and dihydroxyacetone phosphate. Since both can be converted to glyceraldehyde-3-phosphate the fructose molecule is metabolized into 2 triose phosphates that bypass the main rate controlling step in glycolysis, a step catalyzed by 6-phosphofructokinase.

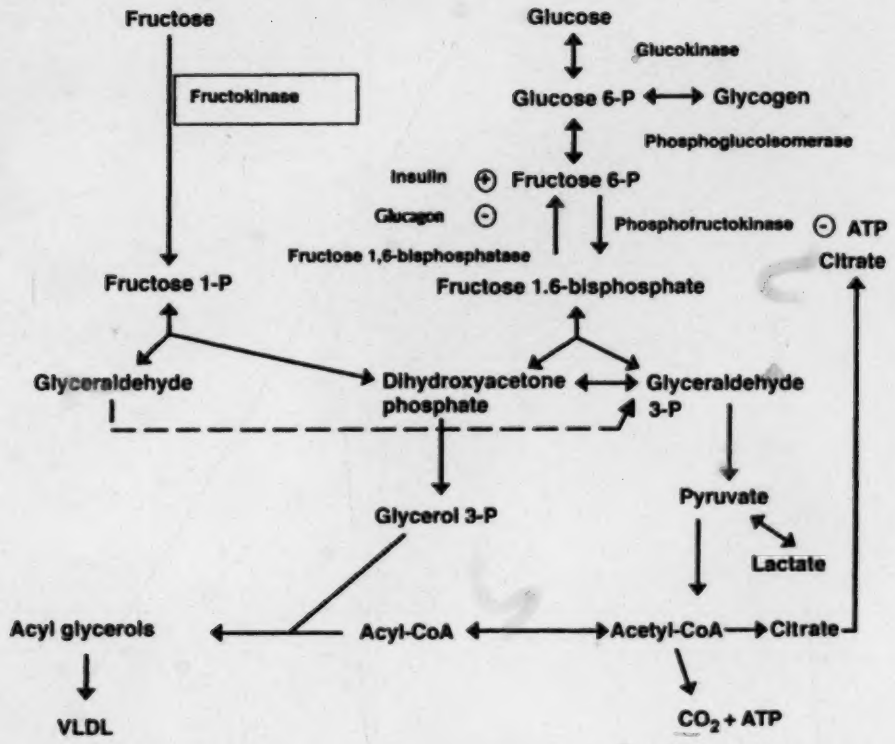


Figure 4. Utilization of fructose and glucose in the liver. Hepatic fructose metabolism begins with phosphorylation by fructokinase (EC 2.7.1.4). Fructose carbon enters the glycolytic pathway at the triose phosphate level (dihydroxyacetone phosphate and glyceraldehyde-3-phosphate). Thus, fructose bypasses the major control point by which glucose carbon enters glycolysis (phosphofructokinase; EC 2.7.1.11), where glucose metabolism is limited by feedback inhibition by citrate and ATP. This allows fructose to serve as an unregulated source of both glycerol-3-phosphate and acetyl-CoA for hepatic lipogenesis. P, phosphate. (Taken from Elliott et al, 2002).

In contrast, hepatic glucose metabolism is limited by the capacity to store glucose as glycogen and more importantly, by the inhibition of glycolysis and further glucose uptake resulting from the effects of citrate and ATP to inhibit phosphofructokinase (Figure 3; Elliott et al, 2002). The products of fructose metabolism in the glycolytic pathway of the liver are glucose, glycogen, lactate, and pyruvate.

4.4 Fructose Feeding in Animal Models

Experiments have been conducted using fructose feeding in the rat (Zavaroni et al, 1980; Kanarek & Gambill, 1982; Bell et al, 1996; Stark et al, 2000). After finding significantly higher plasma insulin concentrations in young rats that were fed a high FR diet for one week (Sleder et al, 1980), the same effects were not observed following acute administration of a FR bolus. Therefore, Zavaroni et al (1980) fed rats (weighing 160-180g) either a high FR diet (66% kcal FR, 22% protein and 12% fat) or normal rat chow for one week. They compared plasma glucose concentrations following either an oral glucose or an oral fructose challenge (180 mg/100 g body weight) and found that mean plasma glucose concentrations were significantly higher in FR rats at 180 minutes. However, significantly higher plasma insulin concentrations were observed at every time point in FR rats. Similarly, plasma insulin concentrations rose in response to an oral FR challenge only in FR rats.

Kazumi et al (1986) studied the effects of dietary fructose on the production of triglyceride in rats (weighing 200-250 g). They made three groups of rats hyperinsulinemic using intraperitoneal insulin pumps and to avoid hypoglycaemia they provided a 10% carbohydrate solution for drinking water. Different sugar was provided

for 14 days to each group: glucose; fructose; or sucrose. Therefore, four groups of control rats were not given exogenous insulin, but provided each of the three sugars and another group of chow fed rats not supplemented with carbohydrate. On day 14, the TG secretion rate was measured and found to be 20% greater in the FR fed rats than in the chow controls, whereas no other differences were observed among the other groups. Similarly, plasma TG concentrations were 120% higher in the FR supplemented group than in chow fed controls. Insulin infusion increased the rate of TG secretion in fructose supplemented rats by 30% over that of the non-insulin infused rats, whereas, insulin infusion decreased the TG concentration rates compared to rats not given exogenous insulin.

Pagliosotti & Prach (1995) fed male rats one of nine diets: three groups were fed a high starch diet (68% kcal) for 8, 16 or 30 weeks, three groups were fed a high sucrose diet (68% kcal) for 8, 16 or 30 weeks and three groups were fed a diet containing 18% kcal from sucrose and 50% from starch for 8, 16 or 30 weeks. Fasting plasma glucose concentrations were similar among the three diet groups at 8, 16 and 30 weeks. However, at 8, 16 and 30 weeks the rats fed the high sucrose diet had significantly higher plasma insulin concentrations compared to low sucrose and starch fed rats. Plasma TG concentrations followed a similar pattern. Those in the high sucrose diet group developed more pronounced insulin resistance than those in the low sucrose diet group based on euglycemic clamps and this worsened over time. These results suggest that both high- and low-sucrose diets can produce insulin resistance in young rats.

A possible mechanism for the development of insulin resistance associated with fructose feeding could be associated with the metabolism of fructose. Fructose uptake by the liver is not inhibited at the level of phosphofructokinase. Infusion of fructose leads to

larger increases of circulating lactate than consuming a similar amount of glucose (Mayes, 1993) and may lead to decreased glucose tolerance by increased non-esterified fatty acid concentrations. Since high-fructose and high-sucrose diets fed to rodents have been used as an animal model for metabolic syndrome it is necessary to study the effects of dietary FR during a sensitive period of development for the endocrine pancreas.

CHAPTER 2: RATIONALE

1. Summary from Literature Review

The early neonatal period is critical for the development of the pancreas and has been shown to be sensitive to the nutrient composition of the suckling diet. Feeding rats a diet high in carbohydrate during the suckling period leads to the development of hyperinsulinemia, the subsequent appearance of glucose intolerance at 35 days and obesity at 55 days. The characteristics persisted into adulthood without any further nutritional insult and offspring of these rats were hyperinsulinemic at birth. FR feeding in young adult rats leads to the development of hyperinsulinemia and the metabolic syndrome. However, this effect can be reversed by changing resuming a normal rat chow. Insulin resistance may be programmed by a high carbohydrate diet during the suckling-weaning transition. Interestingly, there is increased incidence of childhood obesity and the emergence of T2D in childhood corresponds with increased intake of dietary fructose. Does dietary fructose introduced during suckling lead to alterations in whole body glucose metabolism? If so, are these changes accompanied by alterations in organ weight, pancreatic morphology and ß cell response to glucose?

2. Goals

To determine the effects of dietary fructose before weaning on: 1) glucose tolerance, 2) circulating lipid concentrations, 3) body and organ weights, 4) pancreatic morphology, 5) ß cell response to glucose in vitro at 18 days of age in the Sprague Dawley rats.

3. Hypothesis

Fructose feeding during the suckling period will lead to alterations in various aspects involved in glucose metabolism which can be observed at 18 days in the rat.

1. Rats fed FR before weaning will have higher fasting plasma glucose concentrations and secrete more insulin in response to an oral glucose challenge when compared to those fed either by a dam (MF) or rat milk substitute (RMS).

2. Rats fed FR before weaning will have higher plasma levels of TG and NEFA than MF or RMS rats.

3. Since the experimental diets are isocaloric and have similar protein composition to rats' milk we expect rat pups in each treatment group (FR, RMS, MF) will grow at a similar rate and consequently organ weights will be similar.

4. FR feeding leads to hyperinsulinemia in adult rats. Therefore, we expect that FR feeding during a critical period of pancreas development will lead hyperinsulinemia

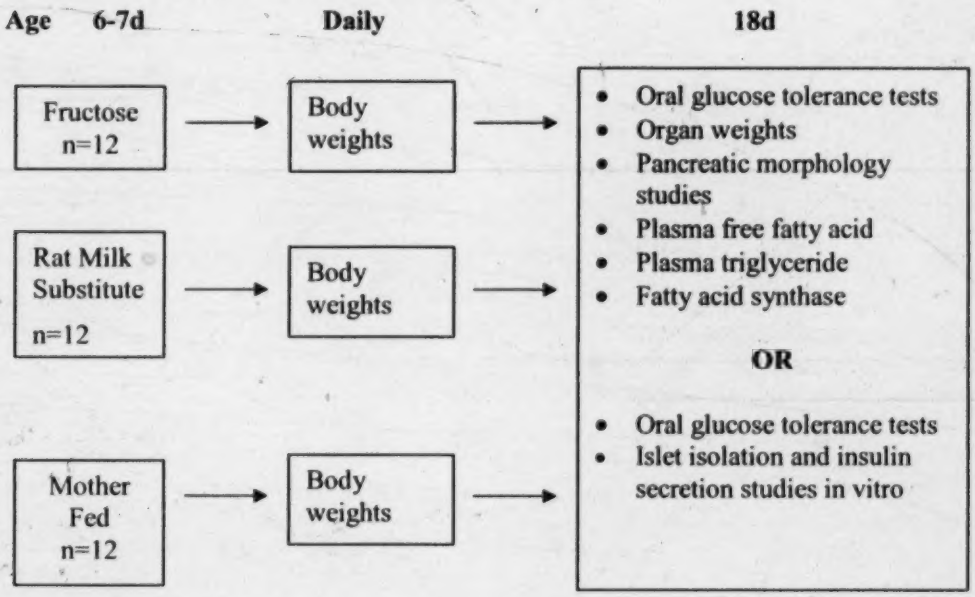
which is maintained by structural changes in the islet, specifically in the insulin producing and secreting ß-cell. Therefore, we expect morphometric studies of pancreata from FR fed rats to show a greater number of ß-cells and larger ß-cells when compared to RMS and MF rats.

5. *In vitro* studies of islets isolated from rat pups fed FR will have greater number of ß-cells and larger ß-cells when compared to RMS and MF rats. ß-cells from FR rats will secrete higher amounts of insulin in response to both high (20 mmol/L) and low (2.6 mmol/L) glucose concentrations in culture.

CHAPTER 3: MATERIALS AND METHODS

1. Experimental Design

Figure 5. Experimental Design: Introduction of Fructose in the Pre-weaning Period



2. Animal Protocols

All procedures used in this study were reviewed and approved by the Faculty of Agriculture, Foresty and Home Economics Animal Welfare Committee at the University of Alberta and in accordance with the Guidelines published by the Canadian Counsel on Animal Care. Sprague-Dawley rat dams with 10 male pups per litter were purchased from either the Biological Sciences breeding colony at the University of Alberta or Charles River Sprague Dawley (Montreal, QC). On postnatal day five, the dams from University of Alberta, with pups were transported to the small animal unit in the Department of Agricultural, Food and Nutritional Science, University of Alberta. Animals that were

purchased from Charles River arrived pregnant and gave birth to their pups in the small animal unit facility.

Ward (1996) reported a post surgery survival rate of 80-90% when gastrostomies were performed on 13 g rat pups compared to 25-50% when 10 g rats were used. Therefore, pups were left with their mothers until they weighed between 11 and 15 g (~ day 7) before the surgeries were performed. On the day of surgery, pups were assigned randomly to one of three diet groups: mother fed (MF), fructose (FR) or rats' milk substitute (RMS). Pups were raised using an artificial rearing technique (described in detail below). Rats were artificially reared on a rat milk substitute (RMS) based diet containing either fructose (FR) or RMS alone from day 6 until weaning on day 18. The macronutrient composition of both experimental formulas was 24% protein, 7% carbohydrate and 69% fat (Table 1) and contained a macronutrient composition that was similar to rat milk.

Table 1. Macronutrient composition and caloric distribution of control formula, fructose formula and rat milk

Nutrient	Control Formula	Fructose Formula	Rat Milk[1]
	g/100mL of milk (% calories)		
Protein	10.0 (24%)	10.0 (24%)	9.4 (24%)
Carbohydrate	2.9 (7%)	2.9 (7%)	3.2 (8%)
Fat	12.7 (69%)	12.7 (69%)	12.0 (68%)

[1]Rat milk composition is taken from Dymsza HA, Czajka DM, Miller SA. Influence of artificial diet on weight gain and body composition of the neonatal rat. J Nutr. 84:100-106, 1964.

3. Diet Preparation

Diets were prepared over a two-day period according to a method described by Ward (1996) and adapted from a proctocol donated by Dr. John Edmond (UCLA). Macronutrient composition of the diet is shown in Table 1], and the specific micronutrient composition of the diet is shown in Table 2. To prepare 1L of diet, a pre-milk base was made by adding Milli-Q deionized water (200 mL; 50°C) to casein (53.6 g) and whey (80.4 g) in a 40:60 casein:whey ratio (Ross Canada, Abbott Laboratories Ltd., St. Laurent QC) to make a smooth paste. The rest of the water (665 mL) was added and gently stirred by hand for 15 minutes to break down any larger lumps. The mixture was stirred on a stir plate for two hours at room temperature, then removed from the stir plate, covered and refrigerated overnight.

On day 2, the milk base was stirred again on the stir plate until it reached room temperature (approximately 45 minutes). Deionized water was added to the milk base to a total volume of 1100 mL, from which 900 mL was removed. The non-calcium mineral mix, carnitine, creatine and ethanolamine (Table 2) were added (in the order given) to the milk base and then mixed using the polytron for approximately one minute, until the entire mixture was homogenous. Calcium dihydroxide, dibasic calcium phosphate and dibasic calcium chloride were added (in that order) to enhance the solubilisation of the calcium salts, followed by the University of Waterloo custom amino acid mixture custom and vitamin diet mixtures (Dyets®, Bethlehem PA) and another supplemental vitamin mixture (Harlan Teklad®, Madison WI). To make the RMS diet, lactose (32.5 g) was used as the source of dietary carbohydrate. To make the FR diet, lactose (16.25 g) and

fructose (16.25 g) were used as the source of dietary carbohydrate and the mixture was stirred.

Table 2. Rats' milk substitute composition

Components	**Amount (g/1.1L)**
Protein [a]	121.8
Supplemental Amino Acid Mixture [b]	0.97
Carbohydrate [c]	32.5
Fat Mixture [d]	140.0
n-3[e]	1.7
n-6	14.6
n-3/n-6 ratio	0.116
Lecithin	11.0
Deoxycholic Acid	0.2
Supplemental Vitamin Mixture [f]	4.0
Noncalcium Mineral Mixture [g]	6.02
$CuSO_4$	0.03
$ZnSO_4$	0.11
Carnitine	0.04
Creatine	0.07
Ethanolamine	0.03 mL
$Ca(OH)_2$	1.5
$Ca(H_2PO_4)_2 \cdot H_2O$	8.0
$CaCl_2 \cdot H_2O$	2.0
Deionized Water	~900 mL
Energy [g]	1.66 kcal/mL

[a] Casein (48.72 g) whey (73.08 g) (Ross Laboratories, St. Laurent QC)
[b] Arginine (510 g/kg), glycine (310 g/kg), taurine (160 g/kg), picolinic acid (20 g/kg) (University of Waterloo Custom Amino Acid Mixture, Dyets ® Bethlehem, PA)
[c] RMS= Lactose, 32.5g or FR= Lactose, 16.25g + fructose, 16.25g (Sigma Chemical Company, St. Louis MO)
[d] Medium chain triglyceride oil (35 g),: coconut oil (67.2 g; Xenex Laboratories, Coquitlam BC), soy (23.8 g; Rapunzel Pure Organics, Valatie NY), olive (14 g; Petrelli, Calgary AB)
[e] n-3 and n-6 contents were estimated according to a fatty acid composition table published by Cordain (2005).
[f] Riboflavin (17 g/kg), niacin (27 g/kg), pyridoxal (14 g/kg), inositol (942 g/kg) (Custom Vitamin Mixture Harlan Teklad ®, Madison WI)
[g] KH_2PO_4 (842 g/kg), $MgSO_4$ (152 g/kg), $FeSO_4 \cdot 7H_2O$ (4g/kg), KI(0.29 g/kg), NaF (0.246 g/kg), $AlSO_4$ (0.156 g/kg), $MnSO_4$ (0.042 g/kg) (Sigma Chemical Company, St. Louis MO)

The oils were melted separately (medium chain triglycerides 35 g; coconut 67.2 g; soy 23.8 g; olive 14 g) and then combined before being added to the milk base. The milk base was stirred by hand and then phosphatidyl choline (1% w/v; 11 g; lecithin; Optimum Health and Vitamins, Edmonton AB) was added to a small volume (200 mL) of the diet, mixed and then added to the total volume of diet (~1.1 L). Phosphatidyl choline was added to keep the diet emulsified. The RMS emulsion was then mixed using a polytron until a proper consistency was achieved (maximum five minutes). Ward et al (1996) used the AR model to produce n-3 fatty acid deficiency in rats. Using a similar design, they fed rats either an n-3 adequate rat milk (n-6 PUFA 12.3 (mg/mL); n-3 PUFA 2.2 (mg/mL); ratio 0.176) or an n-3 deficient (n-6 PUFA 38.6 (mg/mL); n-3 PUFA These diets have previously been shown to provide adequate essential fatty acids to rat pups (Ward et al (1996).

The final emulsion was stirred for one hour (low speed to minimize foaming). The mixture (100 mL) was pipetted into autoclaved bottles, which were then capped and frozen (-20°C) for up to one month. Before using, the bottles of formula were taken from the freezer and placed in a water bath in the sonicator for up to ten minutes. This step thawed the diet and dissolved precipitates that could potentially block the gastrostomy tube. Once thawed, formula was never refrozen, but may have been stored refrigerated for up to one day (4°C) before using.

4. Artificial Rearing Technique

The artificial rearing procedure (AR) was modified from the procedure used by Ward (1996) and originally described by Hall (1975). Diagrams of the gastrostomy tubes and surgical supplies are shown in (Figures 6 and 7). Gastrostomy (G) tubes were made using polyethylene tubing (PE-10; Intramedic ™ Clay Adams Becton, Dickinson and Company (BD), Sparks MD). One end of the tubing was put into a flame for 1s and then flattened (to make a flange) using a solid surface. A washer for the internal portion of the G-tube was hole punched from a plastic Ziploc® bag (SC Johnson, Racine WI), and was guided down the length of the G-tube using a wire (0.01 x 3 inch stainless steel rod; A-M Systems Incorporated Sequim WA) to pierce the washer so that the washer was flush with the flange. The flange and washer help keep the G-tube from coming out of the stomach (Figure 6). G-tubes were made in advance and stored in 70% ethanol. The wires used to exteriorize the G-tube were housed in a Silastic® tube (0.020" ID x 0.037" ID;Dow Corning, Midland MI) and stored separately in 70% ethanol). The Silastic® tube was used to protect the esophagus from being injured by the wire. Larger tube washers (cuffs; Figure 7), used on the exterior portion of the G-tube, were made using polyethylene tubing (PE50; BD, Sparks MD) which had been placed in a flame for 1s, and flattened using a solid surface. Cuffs were used over the G-tube to secure the G-tube at the stomach and at the scruff of the neck. Two sets of cuffs and washers were assembled and put on wires (Figure 6) and stored in a small volume of ethanol (70% v/v).

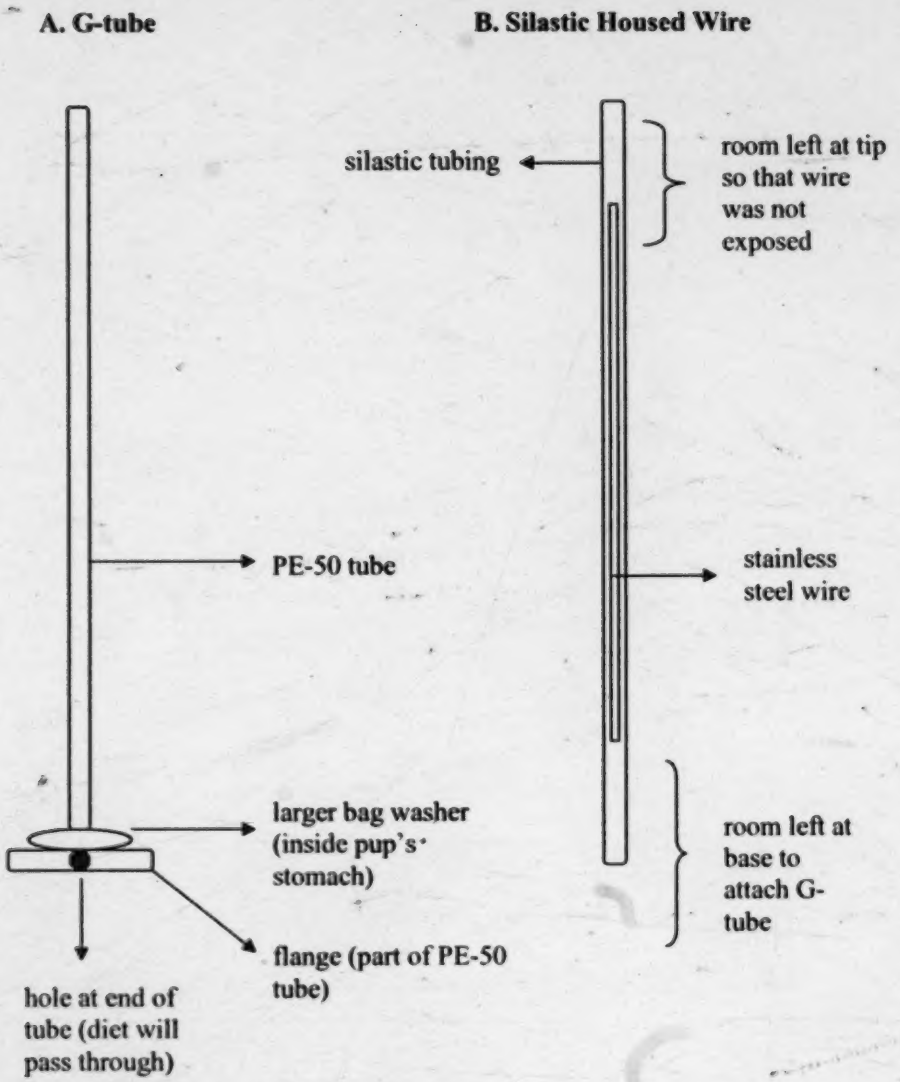

A. G-tube
B. Silastic Housed Wire
silastic tubing
room left at tip so that wire was not exposed
PE-50 tube
stainless steel wire
larger bag washer (inside pup's stomach)
room left at base to attach G-tube
flange (part of PE-50 tube)
hole at end of tube (diet will pass through)

Figure 6. Diagrams of G-tube and silastic housed wire

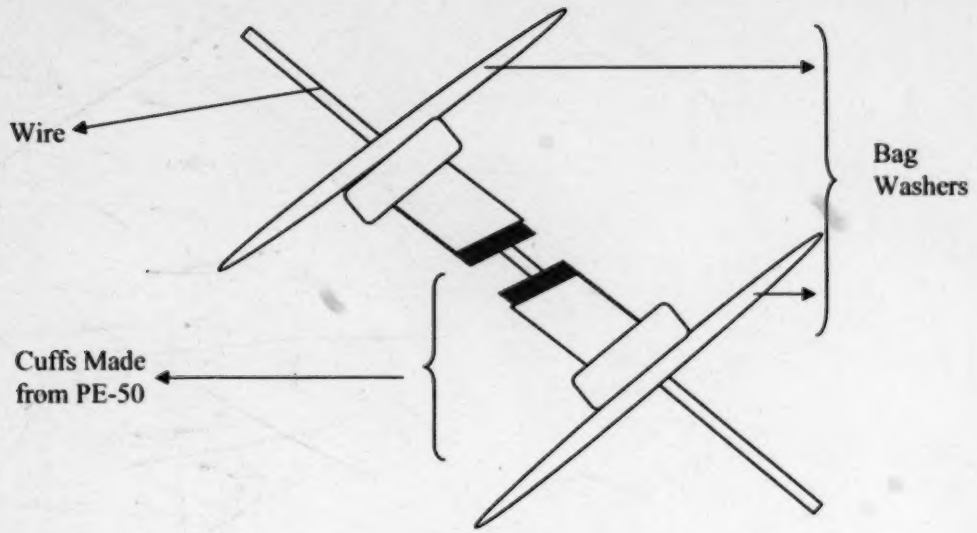


Figure 7. Diagrams of cuffs and washers

4.1 Surgery

4.1.1 Pre-Surgery

At least one hour before surgery, suckling rat pups weighing approximately 13 g (6 d) were taken away from their nursing mothers, put in a plastic mouse cage with autoclaved wood chip bedding, and kept on a heating pad (low setting; Sunbeam Products Incorporated, Delray Beach FL).This short period of fasting was used to reduce the risk of post surgery ileus and decrease abdominal pressure so that stomach contents were less likely to leak into the viscera.

G-tubes were assembled by inserting the silastic-housed wire into the non-flange end of the G-tubes. The wire, inside the silastic, was required to exteriorize the G-tube

while leaving the flange and the internal washer inside the stomach. A second silastic-coated wire was prepared with two sets of PE-50 washers and bag washers. One set of washers was to secure the G-tube where it exteriorized (abdomen) and the other at the neck. Isoflurane (1 mL) was transferred using a transfer pipette, to a 5 mL syringe barrel (BD; Franklin Lakes, NJ) lined with gauze and sealed with parafilm. This was kept inside a chamber that was ventilated with outside air.

4.1.2 Surgery

The pup's nose was held into the syringe and was kept there until surgical plane was achieved and breathing had ceased for 10 seconds (approximately 1 min10 s). Pups were then removed from the isoflurane and then allowed to breathe room air for the remainder of the procedure. Pups were carefully monitored throughout the surgical phase and notes were kept on the progression of the surgery, including time taken for surgical plane to be achieved.

The pup's stomach was located as it is easily seen through the skin. Before the G-tube was inserted into the mouth, it was lightly lubricated with medium chain triglyceride oil, and the distance from mouth to stomach was estimated by aligning the G-tube-wire apparatus with mouth and stomach and this distance was marked. This helped to ensure that the G-tube was not guided into the small intestine or exteriorized before passing into the stomach. The G-tube-wire apparatus was inserted inside the mouth of the rat pup, down the esophagus, inferior to the lower esophageal sphincter and exteriorized by poking the silastic-coated wire out of the stomach, distal to the pyloric sphincter and slightly dorsal and medial to the animal's left upper quadrant.

The silastic-coated wire was gently pulled until the internal washer and flange were flush with the dermis. This procedure took approximately 1 min from the time the animal reached surgical plane. The wire and silastic were disconnected from the G-tube and Neosporin® was applied to the exit wound. The second wire (external washers and cuffs) was attached to the exterior portion of the G-tube and the first set of cuffs and washers was guided down the tube until it was firmly flush with the skin on the abdomen. A drop of Crazy Glue® was carefully applied to keep the G-tube in place. Using the wire, the second set of washers was guided down the G-tube to a position approximately 3 cm from the first set of washers. The wire was pushed into the G-tube and the G-tube was anchored at the 'scruff' of the neck using a set of washers and cuffs, and glued in place.

4.1.3 Recovery

During recovery from the surgery rat pups were monitored for the return of normal heart rate, breathing rate and righting reflex. Sterile, deionized water (0.5 mL) was delivered via the G-tube to all pups. Although pups maintained a faint heart beat through the surgical phase, the return of a normal breathing rate usually required a drop of doxapram and it was applied sublingually. To maximize pup response, an open airway and open mouth were maintained and the pup was placed with the other pups. Pups were 'tested' with water approximately 15 minutes post surgery and if a reaction such as discomfort, noise and/or aspiration were observed, the pup was closely monitored before being housed. At the end of the recovery phase the survival rate was approximately 80%.

4.2 Animal Housing

Nursing dams reared mother-fed (MF) pups in a litter of 10 pups and were housed in individual plastic cages and maintained under a 12-hour dark/light cycle at 21°C. These animals had free access to laboratory rodent diet (5001 LabDiet; Purina Mills Incorporated, Richmond IN).

AR pups were kept warm and in a group immediately post-surgery until they recovered sufficiently. Following recovery, AR pups were given another 3 hours of recovery before feeding was initiated to prevent ileus secondary to anaesthesia. During this period pups were housed in plastic cups (40 oz; Sweetheart ® Cup Company, Owings Mills, MD) which were kept warm (37°C) by 'floating' them in a aquarium. Plastic cups were weighed down by placing weights in the bottom. These were covered with autoclaved corn-cob bedding before the pup was placed in the cup. The cups were immersed in the aquarium to a depth such that the where the pups were in the cup was below the surface of the water. The aquarium temperature was maintained at body temperature (37°C) using immersible compact heaters (low setting; Hagen ® Rolf C. Hagen Incorporated, Montreal QC) and water pumps were used to circulate the heated water. Six pups in cups were placed in each aquarium (maximum18 pups per trial) and the cups were kept in place using a plexiglass lid that fit the aquarium. Cups and bedding were replaced at least twice during the trial and bleach was added to the water in the aquarium to prevent microbial growth.

4.3 Diet Administration

AR pups were fed either RMS or FR via a syringe (10cc Luer ® Lock; BD; Franklin Lakes, NJ) attached to the G-tube. A syringe pump (Harvard Apparatus Incorporated, St. Laurent QC) was used to lock the syringes in place and deliver the diet at an intermittent rate (10 minutes every hour for a 24-hour period). Syringes were fitted with blunted needles (to avoid puncturing the feeding tube) (22G1$^{1/2}$"; BD Franklin Lakes, NJ) and were attached to a piece of tubing (PE-50; BD Sparks MD) that securely fit the G-tube (PE-10; BD Sparks MD) and fastened with tape. Syringes were discarded and replaced daily, needles and feeding tubes were washed, rinsed and dried overnight in an oven (40°C ; Isotemp Fisher Scientific®, Fairlawn NJ) and were changed daily.

Feeding rates were determined each day according to the weight of the MF control pups and based on protocols used in the AR literature (Ward, 1996). The volume of diet to be administered each day was calculated by multiplying the average weight of the MF pups by the appropriate percentage (29% day 1; 31% day 2; and 33% day 3 on) and the feeding rate (cc/min) was calculated by dividing the volume (cc) by feeding minutes (240 min) per day.

4.4 Animal Care

Each morning pup weights were measured and recorded. G-tubes were flushed with sterile, deionized water (0.1 mL) and cleaned every 12 hours. Young rat pups are not able to defecate or urinate on their own and the rat dam promotes defecation and urination by licking them. We were able to stimulate these responses by stroking AR pups in the urogenital region using a warm, wet tissue. Pups were carefully monitored for

signs of distress, growth, and feeding tube quality. Any important observations pertaining to their care were noted. Signs of distress included discoloration of the abdomen, large distended stomachs, labored breathing and pallor. Any pup displaying a sign of distress was euthanized.

Growth was monitored as change in weight and was measured one to two times per day. If the AR pups did not gain weight on the same trajectory as the MF pups, then several steps were taken to ensure that they would recover. Maintaining the pump system required troubleshooting that mostly involved kinked G-tubes or feeding tubes that resulted in lost diet, stalled pumps and/or eventual disconnection from the needle. These situations were corrected by disconnecting the pup from the feeding tube, flushing the G-tube with water to clear blocked lines, taping over the kink to keep the tube patent or cutting the kinked piece of tube, and reattaching the G-tube to the feeding tube. Second, in some cases pups were fed for 12 minutes instead of ten. Third, despite regular gluing, at approximately day 14, G-tubes had popped out of most pups' stomachs. In this case a cheek tube was inserted as an alternate feeding route. Cheek tubes were inserted in pups as young as nine days. Pups younger than nine days were not given cheek tubes since they cannot drink; therefore, the risk of aspiration is greater. The cheek tube surgery is identical to the gastrostomy surgery, except that the tube is poked through the cheek as opposed to down the esophagus and through the stomach.

5. Oral Glucose Tolerance Tests

Oral glucose tolerance tests (OGTT) were performed on fasted rats (4 hours) at day 18 on all animals. AR rats were given water instead of diet during their fast to ensure that they would be well hydrated at the start of the procedure. The glucose challenge (0.3 g/kg body weight for low dose; 3 g/kg body weight for the regular OGTT) was given orally (18G gavage needle) as glucose solution (75% w/v). Tail vein blood samples (approximately 0.1 mL) were collected using a needle (26G; 1"; BD Franklin Lakes, NJ) to draw a blood sample from the tail vein. Blood samples were collected at 0, 15, 30, 45, 60 and 90 minutes post glucose load into sodium fluoride, heparinized tubes (less than 10 IU per mL plasma) and placed immediately on ice. Animals were kept warm using a heating lamp and were placed under a hotter lamp for 10 minutes before the sample was taken. Pups were monitored closely and were removed from the heat if they showed signs of heat stroke or dehydration. The samples were separated using a microcentrifuge (2400 RPM; centrifuge 5415; Eppendorf ® Brinkmann Instruments Incorporated, Westbury NY) for approximately five minutes. Plasma was collected and put into two separate microcentrifuge tubes, one for the insulin assay and the other for glucose, TG and NEFA assays.

The plasma samples were kept frozen (-20°C) until assays were preformed. Plasma insulin concentrations were determined using a Sensitive Rat Insulin Radioimmunoassay (RIA) kit (LINCO Research Inc, St Charles MO USA). Plasma glucose concentrations, NEFA and TG were assessed spectrophometrically using kits: Sigma Trinder Kit (Sigma Diagnostics®, St Louis MO), plasma NEFA (NEFA C; Wako Chemicals, Richmond, VA) and plasma TG (Triglyceride (INT), Sigma Diagnostics ®,

St Louis MO). Samples were pipetted into microplates and were spectrophometrically analysed using a microplate reader (SpectraMax® 190; Molecular Devices Sunny Vale CA) and microplate analysis software (SoftMax® Pro; Molecular Devices Sunny Vale CA).

6. Organ Weights and Tissue Sampling

On day 18 animals were randomly assigned to one of two groups. Animals in the first group were euthanized following the OGTTs, their organs were weighed, and tissue samples (liver, cardiac puncture and mucosal scrapings) were taken for further study. Animals in the second group were maintained on their diet overnight, and the following day they were euthanized, the pancreata were removed and the islets were isolated for further studies.

Animals in the first group were fed (rat dam or AR) for 1-2 hours after the OGTTs. Following re-feeding, pups were given a lethal dose of euthanyl (100 µg/ 100g body weight; intraperitoneal injection). After surgical plane was established, a midline incision was made through the visceral peritoneum. Cardiac puncture was performed on each rat and approximately 0.3 mL blood was taken using a syringe (1mL) fitted with a needle (22G $1^{1/2}$"; BD Sparks MD). Whole blood was put into heparinized tubes (less than 10 IU per mL plasma) and stored on ice before it was separated from the plasma by centrifugation (5 min; 2400 RPM; centrifuge 5415; Eppendorf ® Brinkmann Instruments Incorporated, Westbury NY). Plasma was stored frozen (-20°C) in, non-heparinized tubes until assayed for insulin, TG and NEFA.

Following the cardiac puncture, organs were excised and the weights of the kidneys, spleen, fat pad, pancreas, small intestine and liver were recorded. Samples were prepared from the pancreas, small intestine and liver for further study. The small intestine was removed, flushed with ice-cold saline (0.9% sodium chloride w/v) and divided into three equal lengths. Liver was removed and placed in a labeled tube and snap frozen (liquid N_2) and stored frozen (-70°C) also until the assay for fatty acid synthase activity (FAS) was performed.

7. Lipogenesis: Fatty Acid Synthase

The fatty acid synthase (FAS) assay involved three steps 1) grinding and homogenization of the liver tissue, 2) fatty acid synthase activity determination and 3) bicinchoninic acid protein (BCA) assay. Keeping the samples frozen at all times during the grinding procedure minimized degradation of fatty acid synthase by proteolytic enzymes.

The equipment for grinding samples (screw, plastic container, basin, forceps, spatula and mircocentrifuge tube) was kept on dry ice and was immersed in liquid N_2 before touching the sample or sample tube. Liver tissue was prepared for homogenization by hammering a frozen screw (flat end) on the frozen sample. Samples were sufficiently broken up to a coarse powder. Once the sample was prepared, a frozen tube (15 mL; Eppendorf ® Brinkmann Instruments Incorporated, Westbury NY) was weighed and zeroed. Frozen liver tissue was weighed (1.1-1.2 g) and the remaining sample was returned to the freezer (-70°C).

The next day, cold (4°C) homogenization buffer (2-3 mL; Tables 3, 4 and 5) was added to the sample and kept on ice. Samples were homogenized (polytron; for 30 seconds) and then centrifuged (11,000 RPM; *15,000g* 4°C; 15 min). The supernatant was collected (minimizing the fat) and transferred to an ultracentrifuge tube. Samples were spun (40,000 RPM; *146,000g*; 4°C; 75min; Beckman 50 TI) and the supernatant was transferred to another centrifuge tube.

Table 3. Stock solutions used for the homogenization buffer

Stock Solutions	Preparation
DTT (100 mmol/L)	0.1542 g in 10 mL water
PMSF (500 mmol/L)	0.4355 g in 5 mL water
Soybean Trypsin Inhibitor	10 mg/mL (w/v) water
Aprotinin	5 mg in 5 mL DMSO
Leupeptin	5.3 mg in 5 mL water
Pepstatin A	5 mg in 5 mL water

Table 4. Homogenization buffer stock solution preparation

Reagent	Concentration	Amount (g)
Tris HCl	0.05 mol/L	3.94
Mannitol	0.25 mol/L	22.78
EGTA	1 mmol/L	0.1902
EDTA	1 mmol/L	0.1463
Sodium Pyrophosphate	5 mmol/L	1.1153
Sodium Flouride	50 mmol/L	1.049
*bring up to 450 mL with water pH 7.5 at 4°C		

Table 5. Working homogenization buffer

Reagent	Volume
Homogenization buffer (pH 7.4)	45 mL
DTT stock	500 µL
PMSF stock	100 µL
Soybean Trypsin Inhibitor stock	20 µL
Aprotinin stock	200 µL
Leupeptin	200 µL
Pepstatin stock	200 µL
*make up to 50 mL with water	

The remaining supernatant was transferred to a clear centrifuge tube and polyethylene glycol (PEG 8000; 50% w/v) was added (final concentration of 6%). The tubes were vortexed (2 min) and then centrifuged (9,100 RPM; *10,400g* 4°C; 10 min), the supernatant was discarded and the pellet was gently resuspended in a resuspension buffer (200 µL; Table 6) using a glass rod. Three aliquots were taken from the resuspended pellet (20 µL for protein determination; 80 µL for FAS activity; 80 µL for backup) and stored frozen (-20°C) until further analysis.

FAS activity was determined by combining the enzyme extract (50 µL) and pre-incubation buffer (Table 7) obtained in the grinding and homogenization step (step 1). This mixture was then incubated (37°C) for 30 minutes. Buffer mixture (Table 7; 900 µL) was added and put into a spectrophotometer (heated to 37°C). Double distilled water (50 µL) was put into the reference cuvette and the enzyme sample (50 µL) was put into the second cuvette and was gently mixed. The spectrophotometer was set to zero. The reference position was recorded using the reference cuvette and the measuring position was recorded using the second cuvette. Incubation buffer (Table 7; 50 µL) was added to

the sample cuvette and % absorbance was read (340 nm). The slope of the absorbance curve was recorded as disappearance of glycerol. Bicinchoinic Acid Protein Assay concentration was determined according to kit instructions (Cu_2S; Pierce Biotechnology Incorporated, Rockford IL).

Table 6. Resuspension buffer

Reagent	Concentration	Amount (g)
Tris HCl	0.1 mol/L	0.394
EDTA	1 mmol/L	0.0073
EGTA	1 mmol/L	0.0095
Sodium flouride	50 mmol/L	0.0525
Sodium pyrophosphate	5 mmol/L	0.0550
Sodium azide	--	0.005
Soybean Trypsin Inhibitor stock	--	10µL
Aprotinin stock	--	100 µL
Leupeptin stock	--	100 µL
Pepstatin stock	--	100 µL
*make up to 25 mL with water		

Table 7. Fatty acid synthase activity determination solutions

Solution	Reagents	Concentration
Pre-incubation Buffer	KH_2PO_4	2.7218/10 mL
	DTT	0.0308g/10 mL
Buffer Mixture	1. KPB stock (25 mL)	--
	2. Acetyl CoA (10 mL)	0.35 mM
	3. Malonyl CoA (5mL)	2 mM
	4. B-ME (5 mL)	20 mM
	5. DD H_2O (45mL)	--
Incubation Solution Stock	1. 100 mL EDTA Buffer (KPB):	--
	KH_2PO_4 (27.218g)	2mM
	EDTA (0.116g)	4mM
	2. Acetyl-CoA *	0.35 mM
	3. Malonyl CoA *	2mM
	4. NADPH * (kept on ice)	4mM
	* Solutions kept frozen	

8. Immunohistochemistry

8.1 Fixation and Embedding of Pancreas

Pancreata were carefully removed from the surrounding tissue rinsed in saline (0.9% w/v sodium chloride), dried on gauze, weighed, spread out and placed into labeled, histological cassettes (Histoprep® Omnisette ® Fisher Scientific, Pittsburgh PA). The cassettes were placed immediately in Bock's fixative (25 mL 37% formaldehyde; 75 mL distilled water; 5 mL glacial acetic acid) for 48 hours (21°C). They were washed in four or five rinses of PBS (pH 7.4) to remove fixative, and then stored overnight (PBS pH 7.4;

4°C). The next day pancreata were washed in 4 or 5 rinses of ethanol (70% v/v) and stored in ethanol (70% v/v; 21°C) until they were embedded in paraffin.

Pancreata were prepared for embedding following an immersion schedule (Table 8). Cassettes were immersed in each solution at room temperature (4 cassettes per cycle; in fumehood), and gently agitated at least twice while in each solution. During the second xylene immersion, two beakers containing paraffin (Surgipath® Medical Industries Incorporated, Winnipeg MB) were heated on a hot plate (low setting) in the fume hood. The larger beaker (1 L) had a metal rack (to separate samples), and the smaller beaker (250 mL) had only paraffin and was used to pour into the molds. After the second xylene immersion, cassettes were opened and the pancreata were placed in separate positions (for sample identification) in the metal rack in the larger beaker with melted paraffin. After 30 minutes had elapsed, pancreata were taken from the paraffin and placed in plastic preheated embedding molds (Simport Plastics Limited, Beloeil QC) and filled with paraffin and cooled on ice (~20 min) until the wax was transluscent.

Embedded pancreata were sectioned in Dr. G. Korbutt's lab (Surgical Medical Research Institute, University of Alberta). Two sections per pancreas (4-6 μm) were cut from the paraffin mold and mounted on a slide (Histobond® Fisher Scientific, Pittsburgh PA) and three slides were made per pancreas. Of the three slides cut per pancreas, one slide per pancreas was stained and one section per pancreas was a control section.

8.2 Immunohistochemistry Technique

Pancreas sections were prepared for staining using immunohistochemistry (Table 8) by first removing the paraffin. Slides were placed in an oven (60°C;10 min) and then

placed in coplin jars (Electron Microscopy Sciences, Hatfield PA) to remove additional paraffin (in the fumehood) using xylene (3 X 5 min) sections were then gradually rehydrated (100% ethanol v/v (3 X 2 min); 90% ethanol v/v (1 X 1min); 70% v/v ethanol (1 X 1 min; 10% hydrogen peroxide:methanol v/v (6 min); tap water (3 min).

Table 8. Dehydrating, clearing and embedding rat pancreas in paraffin

Condition	Immersion Time
95% Ethanol (v/v)	1 hr
100% Ethanol (v/v)	30 min
100% Ethanol (v/v)	30 min
Xylene	30 min
Xylene	30 min
Melted Hot Paraffin	30 min

Slides were placed in a moist chamber to prevent drying out. The back of each slide was wiped off, the section was outlined with a hydrophobic pencil and then PBS (1 drop) was applied to prevent the section from drying out. Once PBS had been applied to each slide, it was suctioned off and the blocking serum was added (~50 µL; 20% heat inactivated normal goat serum) to each section, and then the slides were covered with foil and left at room temperature (15 min).

The blocking serum was suctioned off and the primary antibody (guinea pig anti-insulin;1:1000) was added to one section on each slide, to the other section (negative controls) blocking serum was added (25 µL 20% normal goat serum; 4975 µL diluting buffer) and the slides were covered and refrigerated (4°C) overnight. The primary antibody was drained off and slides were rinsed in PBS (3 changes in 5 min). Buffer was suctioned off and the secondary antibody (biotinylated goat anti-guinea pig) was applied

(1:200 v/v; 25µL 20% normal goat serum; 4975 µL diluting buffer; 20 minutes 20°C). The secondary was drained off and the slides were rinsed in PBS (3 changes in 5 min).

Next, a drop of avidin biotin complex (ABC; Vectastain® Vector Laboratories Canada, Burlington ON) was added to both sections on the slides and incubated at room temperature (40 min). Slides were rinsed in PBS (3 changes in 5 min). A small drop of 3,3'-diaminobenzidine (DAB) solution (250 µL substrate buffer; 2.25 mL deionized water; 2 drops of DAB chromogen; 1 drop of hydrogen peroxide) using the DAB peroxidase substrate kit (Vectastain® Vector Laboratories Canada, Burlington ON) was added each section and slides were incubated at room temperature (5 min) and then rinsed twice in tap water. Sections were counter-stained in hematoxylin (Sigma Chemical Company, St. Louis MO) and then immediately (< 10 seconds) and rinsed in PBS (several washes; pH 7.4) until the hematoxylin no longer appeared in the buffer.

Sections were gradually dehydrated using the reverse order of the rehydration step: 70% ethanol (1 X 1 min); 90% ethanol (1 X 1 min); 100% ethanol (3 X 2 min) and then placed in xylene (3 X 5 min) to remove the pappen. Slides were then taken out of the xylene and mounting media was applied (one drop; toluene; brand) and a coverslip was carefully secured and the slides were left to dry (overnight; 20°C).

Table 9. Staining procedure for immunohistochemistry

Step	Process	Condition	Time
1	Melt Paraffin	60°C Oven	10 minutes
2	Clearing	Xylene	3 X 5 minutes
3	Rehydration	100% (v/v) Ethanol	3 X 2 minutes
4	Rehydration	90% (v/v) Ethanol	1 minutes
5	Rehydration	70% (v/v) Ethanol	1 minutes
6	Blocking Endogenous Peroxidase Activity	10% (v/v) H_2O_2: Methanol	6 minutes
7	Wash	Tap Water	3 minutes
8	Hydrophobic Outline	Pappen	-
9	Hydration	PBS pH 7.4	-
10	Blocking Buffer	20% (v/v) goat sera in PBS pH 7.4	15 minutes
11	Primary Antibody	Diluted in PBS 1:1000	Overnight 4°C
12	Clearing Antibody	PBS pH 7.4	Rinse 3x in 5 minutes
13	Secondary Antibody	Diluted in PBS	20 minutes
14	Clearing Antibody	PBS pH 7.4	Rinse 3x in 5 minutes
15	Enzyme Complex	ABC	40 minutes
16	Clearing Step	PBS pH 7.4	Rinse 3x in 5 minutes
17	Substrate Complex	DAB solution and H_2O_2	5 minutes
18	Counterstain	Hematoxylin	30 seconds
19	Wash	Tap water	5 mintues
20	Rehydration	70% (v/v) Ethanol	1 minutes
21	Rehydration	90% (v/v) Ethanol	1 minutes
22	Rehydration	100% (v/v) Ethanol	3 X 2 minutes
23	Clearing	Xylene	3 X 5 minutes
24	Mounting	Per mount	-

8.3 Pancreatic Morphometry and Islet Cell Size

Pancreas sections were analyzed using a light microscope (Leitz Dialux compound light microscope; Wetzlar®, Germany) fitted with a digital camera (CoolSNAP, Photometrics® Roper Scientific Canada, Montreal QC) and using digital software (MetaMorph® 5.0, Universal Imaging Corporation, Montreal QC). More detailed instructions can be found in Appendix A. Cells that stained positively for insulin were identified as β-cells and clusters of positive stained cells were identified as Islets of Langerhans. Connective tissue, lymphoid tissue and large blood vessels were included in

the analysis as non-pancreas area to avoid overestimating the contribution of positively stained area to the overall pancreatic mass.

Once the image was correctly acquired (focusing on computer monitor), the image was separated into a blue image (to calculate the area of positive stained cells, islet area) and a green image (to calculate the area of pancreatic tissue, non-pancreas tissue). The thresholding option was used to quantify to cross-sectional areas occupied by islets, pancreas and non-pancreas tissue and to subtract white space not occupied by tissue. To determine area, sections were systematically sampled using pictures from the entire section in 1.5 X 1.5 mm increments. The area to be measured was highlighted using the threshold feature and then measured (Integrated Morphometry Analysis). Area was estimated using automatically by converting from pixels to microns (0.10482 um/pixel; X 25).

Data from each picture was logged in Excel® (Microsoft XP, 2001) using the Data Log option in Metamorph ®. Results were tabulated as islet area (I Area), pancreas area (P Area), non-pancreas area (NP Area). Cells that stained positively for insulin and were 'aggregated' together were called 'β-cell clusters' and were tabulated to report the number of groups of cells that were positively stained for insulin. Since pancreata were not stained for glucagon, it is not correct to call them islets.

Calculations

Total area= I Area + P Area + NP Area

Average I Area= I Area/ # Islets per slide

Beta Cell Fraction (%)= I Area/Total Area

BCM: Beta Cell Mass per pancreas (mg)= BCF x Pancreatic Weight (mg)

9. *In vitro* β-cell Studies

9.1 Islet Isolation

In vitro β-cell studies were conducted according to the protocol developed by Korbutt et al (1996). Twelve Sprague Dawley rats (18 d) were anesthetized (200 µL Avertin; intraperitoneal injection). Once the animals were in surgical plane, ethanol (70% v/v) was applied to the abdomen and a midline incision (lower abdomen to sternum) was made and the abdominal wall was retracted. The bile duct (duodenum proximal to the stomach) was identified (using a microscope and a dissection lamp) and pulled taut, and clamped (hemostats) where it enters the small intestine.

Pancreata were distended by injecting 2.5 mL cold digestion media (1 mg collagenase (Sigma ® Type V) per mL wash media (Table 10) into the bile duct. The pancreata were excised, transferred to a petri dish containing cold digestion media (Table 11) and residual fat, spleen and blood cell tissue were removed. Pancreata from two rats per treatment were pooled and held in a digestion tube (50 mL Falcon® tube).

Table 10. Wash media

Ingredients	Amount
Pencillin-Streptomycin[1]	5 mL
Hepes Powder	2.4 g/L
F-5 BSA	2.5 g
Brought up to 1L with Hank's balanced salt solution (HBSS) Adjusted to pH 7.4 Filtered Before Using	

[1]BioWhittaker Inc. Walkersville, MD

Table 11. Digestion media

Ingredient	Amount
Wash Media	5 mL per pancreas
Collagenase[1]	5 mg

[1]Type V; Sigma

To break down the exocrine tissue, the remaining digestion media was added (3 mL per pancreas). The digest tubes were closed and secured with Parafilm® and placed in a shaking water bath (37°C; Precision Scientific Thelco 85 RPM; 13 minutes), followed by three firm shakes (by hand) of the tube. To stop digestion, the digestion tubes were centrifuged (1000 RPM; *162g*; 1 min; International Equipment Company, USA), the supernatant removed and resuspended with wash media. The tissue suspension was then filtered through a nylon screen (500 µm) into a new tube (Falcon®; 50 mL). The tissue was then washed twice more with wash media (4°C; 50 mL; 1000 RPM;*162g* 1 min). After the last wash the supernatant was removed and the tissue resuspended with cold Euro-Collins Benzamidine (EC-Benz) solution and placed on ice. EC-Benz is a hypo-osmotic, highly buffered solution used to prevent exocrine degranulation. This is important to prevent exocrine tissue from equilibrating at the same density as islets. EC Benz was added to the digestion tube to a final volume of 50 mL and centrifuged (1000 RPM, *162g* 2 min, 4°C). The tissue pellet was then resuspended in EC Benz (1 mL) and transferred to a gradient tube (50 mL; non-graduated polystyrene tube; Falcon). The filtration tube was rinsed with EC Benz (1 mL; Euro Collins Solution Fresenius Medical Care North America, Lexington MA) and transferred to the gradient tube for a total volume of 2 mL EC Benz and tissue.

A discontinuous bottom load gradient was use to separate the islets from exocrine tissue based on density. The most dense EC DEX solution was added first (14 mL ;1.100 g/mL Dextran, Sigma Chemical Company, St. Louis MO; EC Benz; on ice) followed by gentle vortex, followed by slowly layering 6 mL of each consecutively less dense EC DEX solutions (1.085 g/mL; 1.075 g/mL; 1.045 g/mL; EC Benz) over the mixed tissue suspension. The gradient tubes were then centrifuged (swinging brackets; 2,000 RPM; *650g;* 20 min; 4°C; brake off) to allow the islets to equilibrate at the proper density. The purified islets were collected using a 10 mL syringe fitted with a 14G insyte cannula from the interfaces between 1.075/1.085 g/mL and 1.075/1.045 g/mL and transferred to a new 50 mL conical tube containing 25 mL cold wash solution.

Once all the islets were picked from the gradients the conical tubes were brought to volume with cold wash media and centrifuged (4°C; 1,500 RPM; *364g;* 2 min). Since the islets were still in a dextran suspension the supernatant was carefully removed by bringing the volume down to 15 mL (vacuum suction) and washed in wash media (20°C; 35 mL) and centrifuged again (20°C; 1,000 RPM; *162g;* 2 min). The islets were washed four more times in wash media (4°C; 50 mL) and centrifuged (1,000 RPM; *162g*) for gradually shorter periods (first 1 min; second 10 sec; third and fourth up to speed). After the last wash, the supernatant was carefully removed and islets were resuspended in supplemented Ham's F-10 media (Gibco, Burlington Canada; 3 mL; Table 12) and transferred to a culture plate (non-tissue culture treated; Fisher Scientific, Nepean Canada; diameter 50 cm). The purified and cultured islets were incubated at 37°C (5% CO_2) for 24 hrs before the static incubation (glucose challenge).

Table 12. Ham's F-10 media

Ingredient	Amount
Glucose	10 mmol/L
Isobutylmethylxanthine	50 µmol/L
BSA	0.5%
L-glutamine	2 mmol/L
Nicotinamide	10 mmol/L
Penicillin	100 U/mL
Streptomycin	100 µg/mL
Brought up to 1L Ham's F-10 Media (Gibco, Burlington Canada)	
Adjusted to pH 7.4	

9.2 Static Incubation

For assessment of in vitro viability, the isolated islet's secretory response to glucose was determined after 24hrs of tissue culture by using a static incubation assay. The islet fractions were recovered from the culture dishes, washed (no centrifugation – gravity settle only), assayed for insulin and DNA content (200 µL from 2 mL total volume), and aliquots of 50-100 islets (300 µL from 2 mL total volume) were incubated for 120 min in 1.5 mL of Hams F10 (supplemented with 2 mmol/L L-glutamine, 0.5% BSA and either 2.8 mmol/L glucose or 20 mmol/L glucose). After 120 min at 37°C, the medium was assayed for its insulin content. The remaining tissue was collected, dissociated, fixed and observed for cellular composition using immunohistochemistry. The insulin content of the medium was expressed as a percentage of the total content (i.e., tissue plus medium). Stimulation indices were calculated by dividing the amount of insulin release at 20 mmol/L glucose by that released at 2.8 mmol/L glucose.

Samples taken for insulin content were sonicated in 1ml acetic acid (2N acetic acid, 0.25% BSA), and centrifuged (1,500 RPM; *364g;* 15 min). The resulting supernatants were collected and stored at -20°C until assay for insulin content. Prior to

assay 50µl of the acid extract was dried using a Savant model SC110 Speed Vac and reconstituted with 1ml phosphate buffer (3.75 mM NaCl, 10.0 mM NaH_2PO_4-$2H_2O$, 0.% BSA). Reconstituted insulin extracts were assayed via RIA (Linco Research Inc, St Charles MO, USA).

To determine DNA content samples were washed in citrate buffer (150 mmol/L NaCl; 15 mmol/L citrate; 3 mmol/L EDTA; pH 7.4) and stored frozen (-20°C) as pellets. Prior to assay, the cell pellets were resuspended in lysis buffer (1,000 µL; 10mmol/L Tris; 1mmol/L EDTA; 0.5% Triton X-100; 4°C; pH 7.5), and sonicated. Aliquots were assayed in duplicate by diluting them in Tris- EDTA (1 mL; 10 mmol/L Tris; 1 mmol/L EDTA; pH 7.5) and measuring florescence (490 exc./515 em nm) after the addition of Pico Green double stranded DNA reagent (1 mL; 1:200 dilution with DNA buffer; Molecular Probes, Eugene OR). Samples were run in parallel with and diluted in proportion to standard curve (25-1000 ng/mL) using their DNA. To determine DNA content per islet cell, islet aggregates were dissociated into single cell suspensions.

9.3 Islet Dissociation and Fixation of Cells

The remaining media and tissue (~800 µL per well) were collected from the static incubation and combined into one tube (15 mL). The islets were retrieved from the static incubaton by rinsing each well with dissociation media (Table 13). Each islet suspension was washed twice (centrifuged at 1,000 RPM; *162g;* 1 min) and suspended in 10 mL total volume. Each islet suspension was dissociated by gentle agitation at 37°C using a siliconized Pasteur pipette for 7 minutes and an additional 4 minutes with purified trypsin (250 µL; 1.0 mg/mL w/v dissociation media; Table 13;Roche Diagnostics, Montreal QC)

and DNase (Roche Diagnostics, 100 µL; 0.4 mg/mL w/v dissociation media). The dissociation was completed once the islets were no longer clustered and separate cells were visible under the microscope.

Dissociated islets were washed twice (PBS; 15 mL) and centrifuged (1,200 RPM; 2 min). After the final wash, the pellet was resuspended in PBS (300 µL pH 7.4) and alliquoted (50µL x 2) onto marked slides (Histobond ™, Marienfeld, Germany). Two to three slides per pancreas were prepared. Cells were left to adhere to the slide (20 min; 20°C) placed in fixative (Bocks; 75 mL deionized water; 25 mL 37% v/v formaldehyde; 5 mL glacial acetic acid) for 12 minutes and rinsed (3 X PBS) and then stored (4°C) in ethanol (70% v/v) until stained for insulin using immunohistochemistry (Table 9).

Table 13. Preparation of dissociation media

Ingredients	Volume
H_2O	1800 mL
NaCl	15.88 g
$MgSO_4 \cdot 7H_2O$	0.4 g
KCl	0.8 g
Na_2HPO_4	0.284 g
KH_2PO_4	0.120 g
Hepes Powder	4.771 g
D-glucose	1.0 g
EGTA	0.76 g
$NaHCO_3$	0.7 g
BSA (fraction V)	10.0 g
Penn/Strep	10 mL
2. Refrigerated overnight 4°C	
3. Deionized water added to bring to 2L	
4. The pH was set to 7.4	
5. The media was filtered using a 0.22 µm cellulose acetate filter	

9.4 Calculations

Total Insulin Content

$$I_{total} = \frac{\mu_{ins}}{CF} \times DF_A \times DF_{St}$$

Where:

I_{total}= insulin content (ng/2mL)

μ_{ins} = the mean insulin content (mU/L)

CF = conversion factor 25 ng/mU

DF_A= 10 or the dilution factor for the aliquot

DF_{St}= 24 or the dilution factor the static incubation

Insulin Secretion G-50

$$I_{2.8} = \frac{\mu_{2.8}}{CF} \times \frac{1.5_{ml/well}}{(I_{total} \times 0.6_{mL} / 2_{mL})} \times 100$$

Insulin Secretion G-360

$$I_{20} = \frac{\mu_{20}}{CF} \times \frac{1.5_{ml/well}}{(I_{total} \times 0.6_{mL} / 2_{mL})} \times 100$$

Stimulation Indices

$$SI = \frac{I_{20}}{I_{2.8}}$$

Total DNA Content

$$D_{total} = \frac{\mu_{DNA} \times CF_{10}}{CF_{1000}}$$

Where:

D_{total} = DNA content (pg)

μ_{DNA} = the mean insulin content (mU/L)

CF_{10} = conversion factor (10µg/ng)

CF_{1000}= conversion factor (1µg/1000pg)

β-Cell Recovery

$$\beta_{cell} = D_{total} \times \frac{1_{islets}^{10^6}}{7.1_{pgDNA}} \times P_{insulin(+ve)}$$

Where:
$P_{insulin(+ve)}$ = % cells stained positive for insulin

10. Statistical Analysis

All statistical analyses were performed using Statistical Analysis Software (SAS) version 8.1 (SAS Institute Incorporated, Cary NC). Serial measurements such as body weight, plasma glucose concentrations and plasma insulin concentrations sampled during the OGTT were analysed as repeated measures using the PROC MIXED procedure in SAS (version 8.1; Cary NC). The MIXED model allows for the estimation and testing of differences among treatment or time means when experimental errors are correlated (Wang & Goonewardene, 2004). The advantage of using PROC MIXED for analyzing data from repeated measures experiments is that a covariance structure can be fit to the data; whereas the GLM procedure applies a compound symmetry structure to all the data. The covariance structure that fit this data set was the heterogeneous autoregressive structure (arh(1)). Degree of 'fitness' was interpreted using the Fit statistics from the output. Comparisons were made using the differences of least square means (pdiff)

command in SAS. All means are LS Means reported from SAS output and variance is reported using standard error of the mean. Other measurements that were not taken serially were analysed using the General Linear Model (GLM) in SAS (version 8.1; Cary NC).

Statistical model for repeated measures in PROC MIXED

$$y_{ijt} = \mu + \alpha_i + d_{j(i)} + \gamma_t + (\alpha\gamma)_{it}(b + \varphi_j)x_{ij} + e_{ijt}$$

Where:

Y_{ijt} = the body weight, plasma glucose or plasma insulin concentration measured at time t on the jth rat assigned to the ith diet,
μ = the overall mean effect,
α_i = the fixed effect of the ith diet,
$d_{j(i)}$ = the random effect of the jth rat within the ith diet
γ_t = the fixed *t*th time effect when the measurement was taken
$(\alpha\gamma)_{it}$ = the fixed interaction effect between diet and time
b = the common regression co-efficient of initial measurement of x_{ij}
φ_j = the slope deviation of the *i*th diet from the common slope b
x_{ij} = the initial
e_{ijt} = the random error associated with the jth rat assigned to the ith diet at the t

CHAPTER 4. RESULTS

1. Body Weight

Body weights from day 7 to day 18 are shown in Table 14 and Figure 8. There were no significant differences ($p<0.05$) in body weight among the three diet groups until 10 days of age. On day 10, the artificially reared (AR) rats weighed significantly ($p<0.05$) less than MF rats. The biggest difference in body weight between FR and MF rats was on day 14 when FR pups were 3.56 g smaller than MF rats ($p<0.05$). FR rats 'caught-up' to MF rats on day 15 to the end of the study. On day 16, RMS rats weighed 5 g less than MF rats and this was the biggest difference in body weight between RMS and MF rats over the course of the study. Body weights of FR vs RMS rats did not differ significantly until day 16 when RMS rats weighed on average 3.2 g less than FR rats ($p<0.05$), but by day 18 RMS rats and FR rats had similar body weights. Although RMS rats 'caught-up' to FR rats on day 18, they weighed 3.3 g less than the MF control pups ($p<0.05$).

Table 14. Effect of feeding rat pups on body weight (g) from 7 days to 18 days of age.

	Body Weight (g)		
Age	FR (n=17)	RMS (n=18)	MF (n=30)
7	16.2 ± 0.4	16.3 ± 0.4	15.3 ± 0.3
8	17.3 ± 0.5	17.5 ± 0.4	17.6 ± 0.3
9	18.3 ± 0.5	18.6 ± 0.5	19.6 ± 0.4
10	19.3 ± 0.7^{b}	20.0 ± 0.6^{b}	22.0 ± 0.5^{a}
11	21.6 ± 0.7^{b}	21.8 ± 0.7^{b}	24.1 ± 0.5^{a}
12	23.7 ± 0.7^{b}	23.8 ± 0.7^{b}	26.0 ± 0.5^{a}
13	25.7 ± 0.9^{b}	26.0 ± 0.9^{b}	28.4 ± 0.7^{a}
14	27.2 ± 1.0^{b}	26.4 ± 0.9^{b}	30.8 ± 0.7^{a}
15	31.1 ± 1.1^{ab}	28.6 ± 1.0^{b}	32.8 ± 0.8^{a}
16	32.9 ± 1.1^{a}	29.6 ± 1.1^{b}	34.6 ± 0.8^{a}
17	36.5 ± 1.1^{a}	33.3 ± 1.0^{b}	36.2 ± 0.8^{a}
18	38.1 ± 1.2^{ab}	34.9 ± 1.2^{b}	38.1 ± 0.9^{a}

Data is mean ± SE

^{a,b} numbers in same row with different superscripts are significantly different ($P<0.05$)

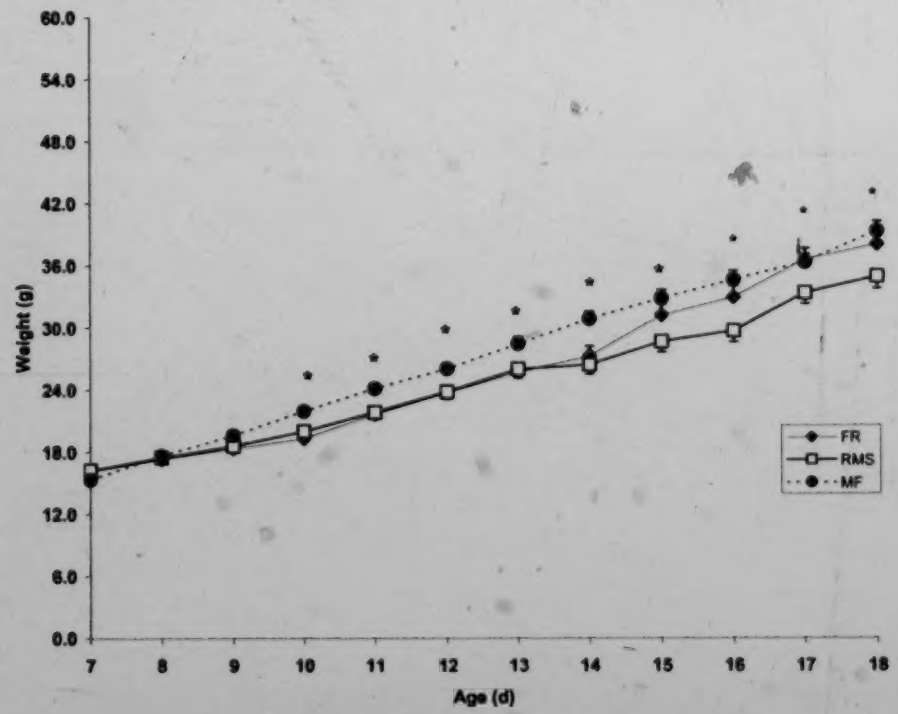


Figure 8. Body weight (g) from days 7-18 in the young rat (* $p<0.05$).

2. Organ Weights

Organ weights are shown in Table 15; both the absolute weight and the weight per 100 g body weight are presented. No differences were observed in pancreatic, liver or fat pad weights among the three diet groups. Kidney weights are reported as total kidney weight (ie. left and right kidney) and no differences were observed among the three diet groups. Spleen weights were significantly higher ($p<0.05$) in both RMS and FR rats compared to MF rats only after controlling for differences in body weight. Small intestine weights, (expressed as an absolute weight) of the RMS rats were the heaviest and were significantly heavier ($p<0.05$) than MF rats. However, small intestine weights did not differ between RMS vs FR rats or FR vs MF rats.

Table 15. Weights of excised organs (g) on day 18 expressed as an absolute value and based on body weight (g/100g).

	Absolute Organ Weight (g)			Organ Weight (g/100g body wt)		
	FR	RMS	MF	FR	RMS	MF
Organ	(n=4)	(n=3)	(n=11)	(n=4)	(n=3)	(n=11)
Pancreas	0.23±0.04	0.24±0.04	0.21±0.02	0.57±0.08	0.62±0.10	0.51±0.05
Fat Pad	0.07±0.01	0.06±0.01	0.07±0.00	0.17±0.02	0.15±0.02	0.16±0.01
Liver	1.64±0.03	1.53±0.06	1.58±0.03	3.99±0.12	3.93±0.14	3.84±0.07
Kidney	0.59±0.02	0.57±0.02	0.55±0.01	1.44±0.05	1.47±0.06	1.33±0.03
Spleen	0.19±0.01	0.19±0.01	0.15±0.01	$0.47±0.03^{a}$	$0.49±0.03^{a}$	$0.36±0.02^{b}$
Small Intestine	$1.88±0.09^{ab}$	$2.26±0.11^{a}$	$1.65±0.06^{b}$	$4.63±0.22^{b}$	$5.48±0.30^{a}$	$4.06±0.14^{b}$

Data is mean ± SE

^{a,b} numbers in same row with different superscripts are significantly different ($p<0.05$)

3. Oral Glucose Tolerance Tests

3.1 Low-dose (0.3g/kg) OGTT

On day 18, there were no significant differences observed in plasma glucose concentrations at baseline among the three diet groups (Table 16, Figure 9). In both MF and RMS rats, plasma glucose concentrations rose to similar levels at 15 minutes post-glucose load, whereas FR rats reached the highest plasma glucose concentration at 30 minutes post-glucose load. Plasma glucose concentration at 30 minutes was 1.3 mM lower in FR compared to MF rats. At 15 and 30 minutes plasma glucose concentrations were significantly higher ($p<0.05$) in MF rats than in FR and RMS rats. Plasma glucose concentrations rose from baseline to 30 minutes by 1.6 mM in MF rats, 1.2 in RMS rats and 0.96 mM in FR rats. There were no statistically significant differences in plasma glucose concentration observed among the three diet groups at 45 or 60 minutes. At 90 minutes plasma glucose concentrations returned to baseline in all groups. No significant differences were observed in the AUC for glucose.

Table 16. Effect of feeding rat pups during suckling on plasma glucose concentrations (mM) after a low dose oral glucose challenge (0.3g/kg).

	Plasma Glucose Concentrations (mM)		
Time (min)	FR (n=11)	RMS (n=12)	MF (n=15)
0	7.2 ± 0.4	7.7 ± 0.4	8.3 ± 0.4
15	8.2 ± 0.4^{b}	8.9 ± 0.4^{ab}	9.9 ± 0.4^{a}
30	8.2 ± 0.4^{b}	8.8 ± 0.4^{ab}	9.5 ± 0.3^{a}
45	8.0 ± 0.4	8.4 ± 0.4	8.8 ± 0.4
60	8.0 ± 0.4	8.6 ± 0.4	8.6 ± 0.4
90	7.5 ± 0.4	8.0 ± 0.4	8.1 ± 0.3
Δ AUC	66.9 ± 35.6	71.1 ± 22.2	54.8 ± 20.5

Data is mean ± SE
^{ab} numbers in same row with different superscripts are significantly different (P<0.05)

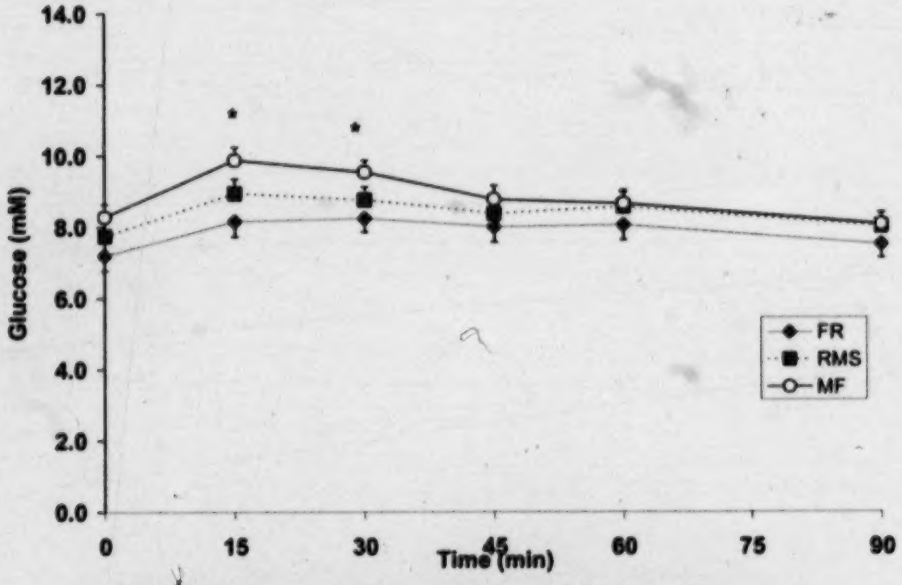


Figure 9. Plasma glucose response (mM) to a low dose oral glucose challenge at 18 days in the young rat (*p<0.05: FR vs MF).

3.2 Regular Dose (3g/kg) OGTT

3.2.1 Glucose Concentrations

Before the regular dose (3g/kg) OGTT, plasma glucose concentrations were significantly lower ($p<0.05$) in AR pups than the MF pups (Table 17, Figure 10). Plasma glucose concentrations at baseline were not significantly different between FR and RMS. At 15 minutes there were no significant differences detected among the treatment groups. AR pups reached maximal plasma glucose concentrations at 30 minutes post-glucose load compared to 45 minutes for MF rats. The plasma glucose concentrations of the RMS rats compared to MF rats were not statistically different at any point during the OGTT. At 30, 45 and 60 minutes FR rats had significantly lower ($p<0.05$) plasma glucose concentrations than MF rats and RMS rats. At 90 minutes plasma glucose concentrations remained slightly elevated and were approximately 1 mM above baseline in all three diet groups and did not differ among groups. No significant difference was observed in the AUC for glucose.

Table 17. Effect of feeding rat pups during suckling on plasma glucose concentrations (mM) after a high dose oral glucose challenge (3g/kg).

	Plasma Glucose Concentrations (mM)		
Time (min)	FR (n=11)	RMS (n=14)	MF (n=20)
0	6.1 ± 0.3^{b}	5.9 ± 0.3^{b}	7.1 ± 0.3^{a}
15	8.8 ± 0.5	9.6 ± 0.4	9.9 ± 0.4
30	9.4 ± 0.5^{b}	11.1 ± 0.5^{a}	11.0 ± 0.4^{a}
45	8.1 ± 0.5^{b}	10.3 ± 0.5^{a}	11.1 ± 0.4^{a}
60	8.2 ± 0.6^{b}	9.9 ± 0.5^{a}	10.3 ± 0.4^{a}
90	7.3 ± 0.4	7.4 ± 0.4	8.0 ± 0.3
Δ AUC	194.4 ± 26.1	302.0 ± 30.4	257.6 ± 36.2

Data is mean ± SE
^{a,b} numbers in same row with different superscripts are significantly different ($p<0.05$)

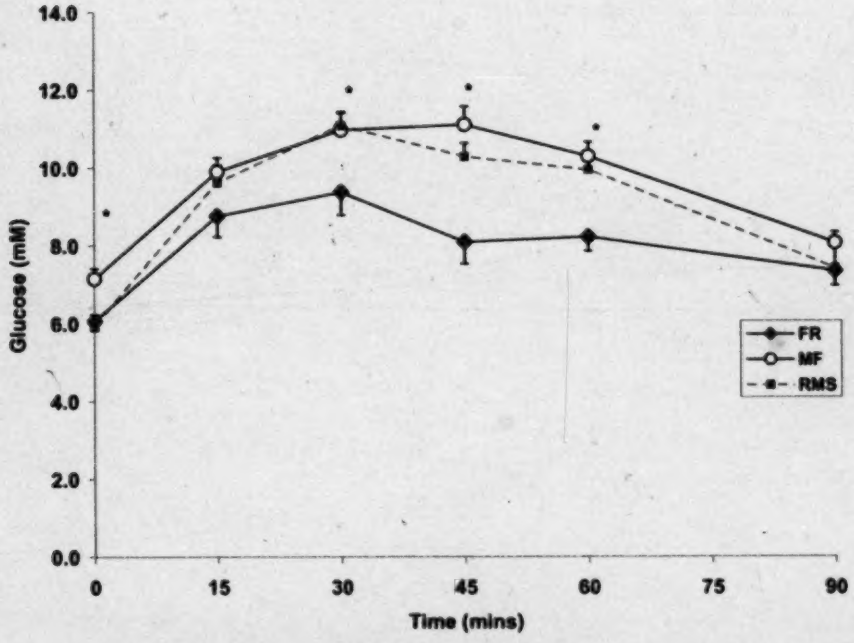


Figure 10. Plasma glucose response (mM) to a high dose oral glucose challenge (3g/kg) at 18 days in the young rat (* $p<0.05$) t=0 MF vs RMS and FR t=30,45,60 MF and RMS vs FR

3.2.2 Insulin Concentrations

Plasma insulin concentrations were similar ($p<0.05$) between the three diet groups at baseline (Table 18, Figure 11). Peak plasma insulin concentrations were observed at 15 minutes in FR rats, whereas RMS and MF rats reached their highest plasma insulin concentrations at 45 minutes post glucose load. Plasma insulin concentrations at 15 and 30 minutes were almost 3 times higher in FR rats compared to RMS rats and approximately two-fold higher than MF rats ($p<0.05$). However, no differences were detected between RMS and MF rats. No further differences were observed at either 45, 60 or 90 minutes. Although not significantly different at 90 minutes, plasma insulin concentrations returned to baseline in FR rats, but remained slightly elevated in RMS and MF rats. No significant difference ($p<0.05$) was observed in the AUC for insulin.

Table 18. Effect of feeding rat pups during suckling on plasma insulin concentrations (ng/mL) after a high dose oral glucose challenge (3g/kg).

	Plasma Insulin Concentrations (ng/mL)		
Time (min)	**FR (n=6)**	**RMS (n=4)**	**MF (n=4)**
0	0.05 ± 0.01	0.04 ± 0.01	0.03 ± 0.02
15	0.21 ± 0.02[a]	0.07 ± 0.03[b]	0.09 ± 0.03[b]
30	0.20 ± 0.04[a]	0.07 ± 0.04[b]	0.11 ± 0.04[ab]
45	0.11 ± 0.02	0.10 ± 0.03	0.13 ± 0.03
60	0.12 ± 0.02	0.09 ± 0.02	0.11 ± 0.02
90	0.04 ± 0.01	0.06 ± 0.01	0.08 ± 0.01
Δ AUC	54.00 ± 18.36	16.87 ± 4.71	30.25 ± 5.53

Data is mean ± SE
[ab] numbers in same row with different superscripts are significantly different ($p<0.05$)

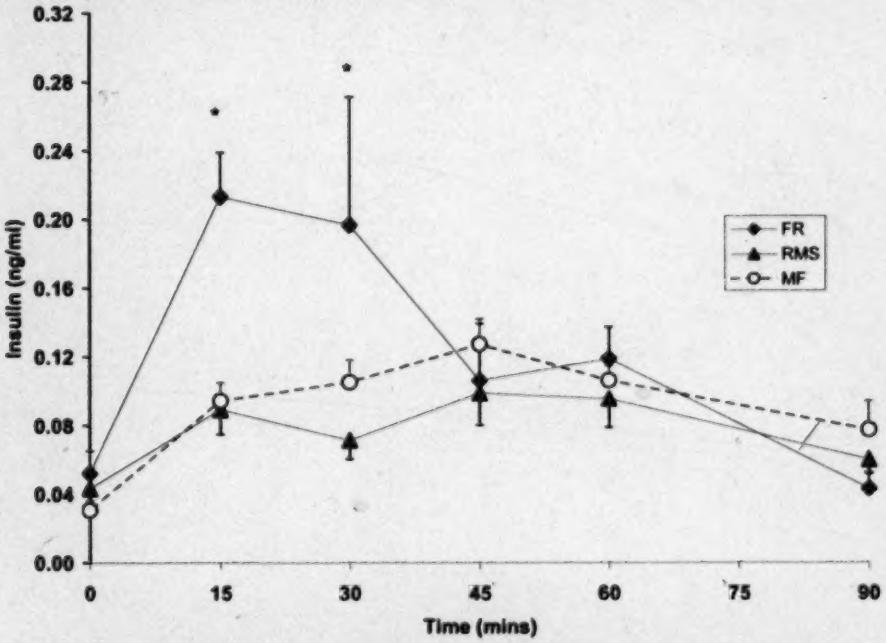


Figure 11. Plasma insulin response (ng/mL) to a high dose (3g/kg) oral glucose challenge at 18 days in the young rat (* $p<0.05$).

4. Isolated Islet Studies

4.1 Insulin Secretion *in vitro*

The effect of feeding FR on insulin secretion under low (2.8mM) and high (20mM) glucose concentrations were studied *in vitro* (Table 19, Figure 12). Replicates were excluded if there was an insulin:DNA ratio of less than 1.6 (ng/pg). This resulted in the exclusion of two FR and two RMS replicates. Significant variation was observed in the results as the study progressed, this was managed by analyzing the effect of day as a block using the GLM procedure (SAS 8.1, Carey NC). No significant difference ($p<0.05$) was observed in insulin secretion under either low (2.8 mM) or high (20 mM) glucose concentrations. Stimulation indices (2.8mM/20mM) were significantly higher in RMS rats compared to both FR and MF rats.

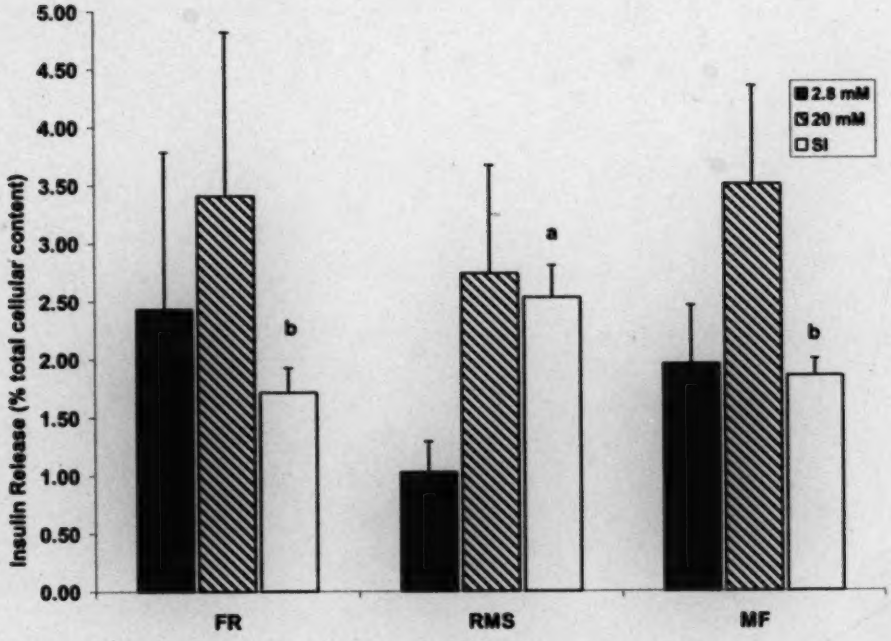


Figure 12. Increase in insulin secretion (%) under low (G50) versus high (G360) glucose conditions and the SI (G360/G50) *in vitro* at 18 days (^{ab} numbers with different superscripts are significantly different ($p<0.05$).

4.2 Isolated Islet Characteristics

DNA content was measured and immunohistochemical analyses of isolated islets were not significantly different ($p<0.05$) among the three diet groups. Further, no differences were observed in DNA recovery or in proportions of α-cells (%) or β-cells (%) among the three diet groups (Table 19). However, FR rats had nearly half the number of β-cells ($p<0.05$) as MF rats and RMS rats (Figure13).

Table 19. Characteristics of islets isolated from rats on day 18.

Parameter†	FR (n=5)	RMS (n=6)	MF (n=7)
Insulin Content (ng)	4092.6± 1302	4918.6 ± 861	5248.6 ± 735
Insulin release: 2.8mM	2.43 ± 1.35	1.02 ± 0.26	1.95 ± 0.5
Insulin release: 20mM	3.41 ± 1.41	2.74 ± 0.93	3.50 ± 0.84
Stimulation Index (SI)	1.71 ± 0.21[b]	2.53 ± 0.28[a]	1.85 ± 0.15[b]
% β-cells	59.0 ± 13.1	55.1 ± 6.5	40.4 ± 4.5
Total ß-cells ($x10^6$ cells)* per pancreas	0.07 ± 0.03[b]	0.16 ± 0.06[a]	0.13 ± 0.05[a]

Data is mean ± SE
†Data is from pooled islets (2 pancreata per replicate)
*Day was significant ($p<0.05$)
[ab] numbers in same row with different superscripts are significantly different ($p<0.05$)

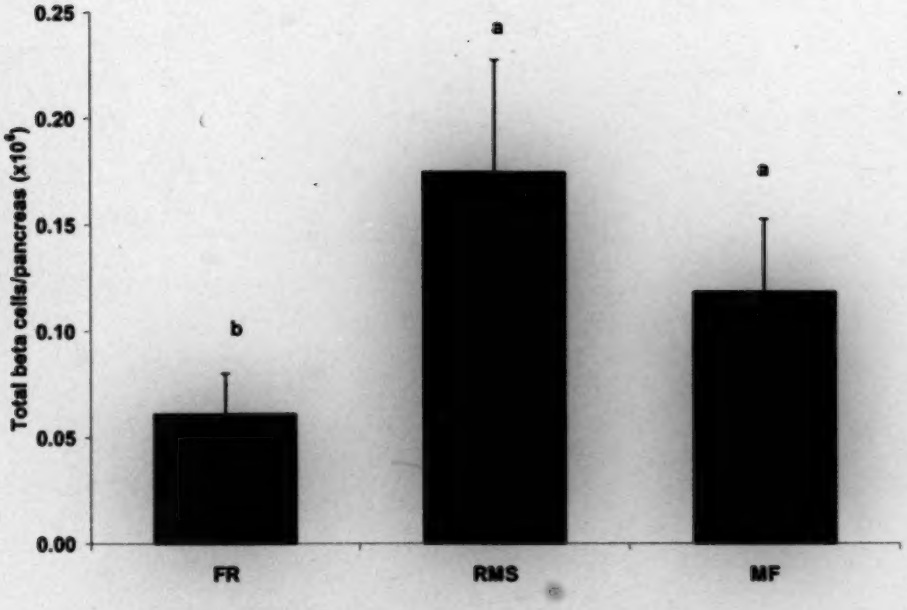


Figure 13. Number of β-cells (x 10^6) at 18 days after isolating islets (^{ab} numbers with different superscripts are significantly different ($p<0.05$).

5. Pancreatic Morphology

There were no significant differences in β-cell properties between the three diet groups as estimated using morphological techniques (Table 20). The number of β-cell clusters, the mean β-cell area, the proportion of β-cells in the pancreas or β-cell fraction and β-cell mass did not differ among groups.

Table 20. Effect of diet on β-cell size (β-cell cluster number, mean β-cell area, β-cell fraction) estimated using microscopic and morphological studies.

Parameter	FR (n=4)	RMS (n=3)	MF (n=9)
# β-cell clusters	93 ± 8	142 ± 26	103 ± 20
Mean β-cell area [+ve ins area: # clusters]	97.71 ± 10.12	102.41 ± 11.26	92.28 ± 52.26
β-cell Fraction %	0.51 ± 0.00	0.63 ± 0.00	0.53 ± 0.00
β-cell Mass (mg)	1.21 ± 0.19	1.15 ± 0.15	1.47 ± 0.31

Data is mean ± SE
[ab] numbers in same row with different superscripts are significantly different ($p<0.05$)

6. End of Study

6.1 Fed State

At the end of the study, under fed conditions, concentrations a variety of metabolic parameters were measured (Table 21). MF rats had significantly higher ($p<0.05$) plasma glucose concentrations than either FR or RMS rats. There were no differences in plasma insulin concentrations among the three groups. While FR rats and MF rats had approximately higher plasma NEFA concentrations than RMS rats, there were no differences between FR and MF rats. Hepatocytes isolated from MF rats had approximately twice the fatty acid synthase enzyme activity (Figure 14) compared to enzyme activities in hepatocytes from RMS or FR rats ($p<0.05$).

Table 21. Effect of diet during suckling on plasma glucose, insulin, non-esterified fatty acid (NEFA) concentrations and FAS activity in the rat at 18 days in a fed state.

Parameter	FR (n=4)	RMS (n=3)	MF (n=7)
Glucose (mM)	9.3 ± 1.3^{b}	7.9 ± 1.2^{b}	13.0 ± 0.8^{a}
Insulin (ng/mL)	0.10 ± 0.09	0.32 ± 0.09	0.20 ± 0.07
NEFA (mM)	2.2 ± 0.3^{a}	0.9 ± 0.4^{b}	1.9 ± 0.2^{a}
Fatty Acid Synthase (mmol/ min mg protein)	6.0 ± 1.8^{b}	5.7 ± 2.0^{b}	10.8 ± 1.1^{a}

Data is mean ± SE
^{ab} numbers in same row with different superscripts are significantly different ($p<0.05$)

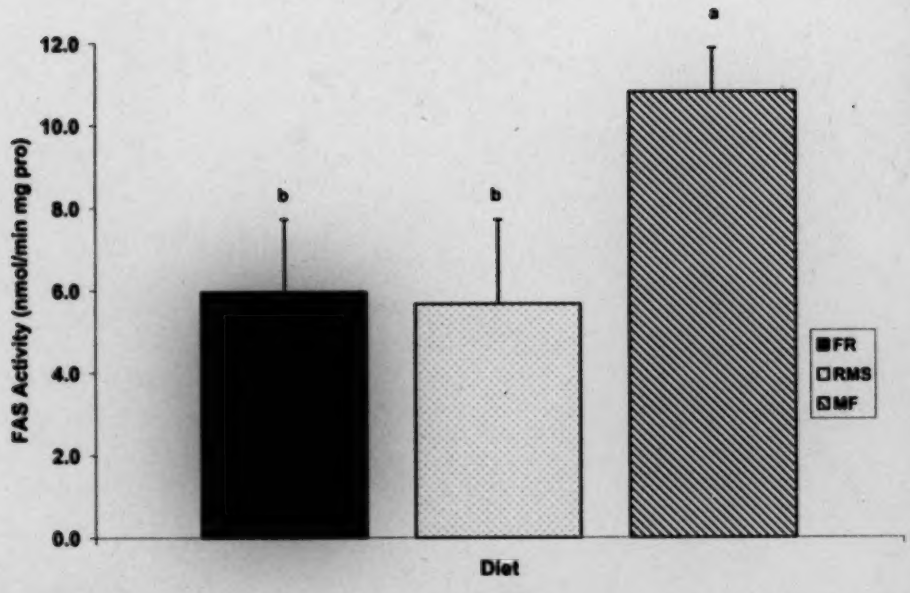


Figure 14. Fatty acid synthase activity (mmol/min·mg·protein) in livers of rats at 18 days ([ab] numbers with different superscripts are significantly different ($p<0.05$).

6.2 TG Concentrations

Plasma triglyceride (TG) concentrations were not significantly different ($p<0.05$) among the three diet groups under either fasting or fed conditions (Table 22, Figure 15).

Table 22. Effect of diet during suckling on TG concentrations (mg/dL) under both fed and fasting conditions in the rat at 18 days.

	Triglyceride Concentration (mg/dL)		
Prandial State	**FR (n=4)**	**RMS (n=3)**	**MF (n=9)**
Fasting	53.5 ± 12.4	74.4 ± 14.3	57.6 ± 8.3
Fed	269.0 ± 50.8	262.95 ± 71.8	383.80 ± 27.8

Data is mean ± SE
[ab] numbers in same row with different superscripts are significantly different ($p<0.05$)

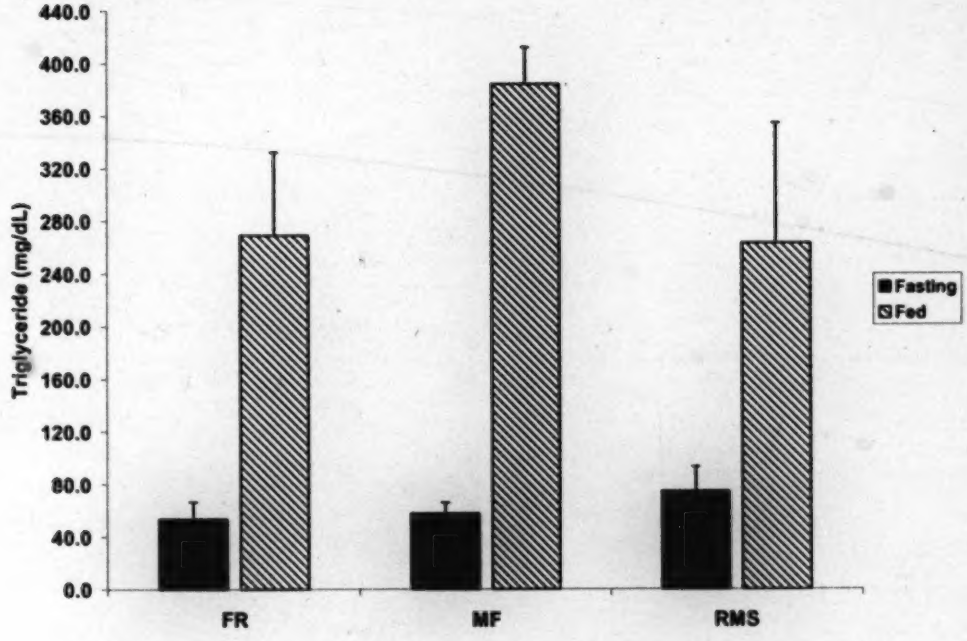


Figure 15. Plasma triglyceride (TG) concentrations (mg/dL) under either fasting or fed conditions in rats at 18 days (^{ab} numbers with different superscripts are significantly different ($p<0.05$).

CHAPTER 5: DISCUSSION

1. Oral Glucose Tolerance Tests (OGTT)

This study was designed to examine the effects of dietary fructose during the suckling period on glucose metabolism in the rat. After 11 days of dietary FR during the suckling period, those in the FR fed group demonstrated an increased insulin secretory response following an oral glucose challenge (3g/kg). This suggests that FR feeding from 4 days to 18 days leads to altered insulin secretion at 18 days, which could predispose to glucose intolerance later in life.

At baseline, plasma insulin concentrations of insulin were similar among the three diet groups. However, the maximum insulin response observed occurred at 15 minutes in the FR group, but at 45 minutes in the other two groups. Further, the maximal insulin concentration reached in FR rats was approximately twice the maximal insulin concentration reached in the other two groups. This suggests that the insulin response to an oral glucose challenge in FR rats is much greater and faster than in rats from the other groups. Therefore, the insulin secretion pattern is affected by FR feeding during the suckling period. Since there were no differences in glucose or insulin responses to an OGTT in the RMS and MF groups, the heightened insulin response in the FR group cannot be explained by stressors involved in AR. Although there were was a markedly different insulin response to glucose, blood glucose concentrations did not differ. Stark et al (2000) conducted OGTTs in adult rats (12 and 15 weeks old) that were fed either a control diet (10 % sucrose, 53% starch, 7% soybean oil), a high fat (10 % sucrose, 35% starch, 25% soybean oil) or a high fructose diet (10 % sucrose, 53% fructose, 7 % soybean oil) for a 3 month period, starting at 6 weeks of age. They reported no significant

differences in blood glucose concentrations at any time during the OGTT in both age groups, among any of the diet groups. This suggests that dietary FR fed after weaning does not lead to changes in glucose tolerance in adulthood. Unfortunately, they did not report the corresponding plasma insulin concentrations for the OGTT. Therefore it not known from Stark et al's study whether insulin secretion is affected by FR feeding in the postweaning period. The present study is one of a few that measures the insulin response to an oral glucose challenge (OGTT) during the pre-weaning period. Balonan & Sheng (2000) performed OGTTs in 20 day old rats that were either malnourished or overfed by raising rats in small (4), medium (10) or large litters (16). They observed significantly lower fasting insulin concentrations in the pups from large litter and higher insulin concentrations in rats from the small litters. This study showed that manipulation the diet of rats during suckling "imposes changes in insulin and glucose tolerance at weaning" (Balonan & Sheng, 2000).

Vadlamudi et al (1993) have shown in the HC model that 24-day old rats fed the HC diet have higher total pancreatic insulin content compared with those that were mother-fed. Since total insulin content was not measured in our study, it's not known whether FR feeding in the suckling period results in an increased insulin content. It is possible that the increased insulin secretion observed in our study reflects an overall increase in insulin available to be secreted in response to a glucose stimulus.

Plasma insulin concentrations are the result of the balance between insulin secretion and removal (Misbin,1985; Vadlamudi et al, 1993) and impairments can result in hyperinsulinemia. Similar to this study, Vadlamudi et al (1993) observed the combination of higher plasma insulin concentrations and increased insulin sensitivity and

therefore, HC rats were glucose tolerant at 18 days. This suggests that early diet can influence insulin secretion at 18 days, but glucose intolerance may not appear until later in life.

2. *In vitro* Insulin Secretion Studies

In addition to examining insulin secretion *in vivo*, insulin secretion *in vitro* was also determined. The insulin response to a low and a high concentration of glucose by isolated islets *in vitro* was similar in rats from all diet groups. Therefore, this is different from what was observed *in vivo*.

There are differences between what we observed as a functional measure of the insulin response to glucose (ie. OGTT) *in vivo* and what was observed *in vitro*. The β-cell response to glucose *in vitro* isolates the secretory response to glucose from other factors that stimulate insulin secretion *in vivo* such as, hormonal feedback involving many organs and tissues. Therefore, it appears that insulin secretory responses to both high and low concentrations of glucose *in vitro* do not change with early FR feeding relative to rats that were MF. It is possible that insulin secretion *in vitro* may not accurately represent the in vivo physiological process.

3. Morphology

Morphological studies of the pancreas were conducted to determine the structural adaptations in the endocrine pancreas (ie. β-cell hypertrophy) that could result from early FR feeding. We found that there were no statistically significant differences in β-cell cluster number, β-cell mass and β-cell area among diet groups.

It could be that we estimated pancreas morphology during a period of endocrine pancreas 'remodelling'. Since the suckling period is marked by a wave of apoptosis that depletes islet β-cells and results in their neogenesis followed by their replacement from pancreatic ductal tissue, it is possible that differences in pancreatic remodelling in FR fed rats can be observed only after this window has passed (Scaglia et al, 1997). Therefore, future studies may seek to understand whether dietary FR affects β-cell apoptosis and neogenesis during this window of remodelling.

Since we characterized pancreatic morphology using only cells that stained positively for insulin, we did not truly approximate islet size. Pancreatic sections were not stained for glucagon-secreting α-cells and were therefore entirely excluded from the analysis. Since the ratio of β-cells to α-cells may differ among diet groups, future studies examining pancreatic morphology should include approximations of α-cell size. Our results are further limited since only one section per pancreas was analysed. However, the purpose was to collect crude information about β-cell characteristics that could not be tested *in vitro.* While we did not include this in our morphometric analysis, we did stain for glucagon in isolated rat islets. Since no significant differences were observed, we would expect similar findings in our morphological studies.

Vadlamudi et al (1993) showed that HC rats had a slightly greater mean islet diameter and more insulin-positive cells per pancreas. However, they found that on day 12, after 8 days of HC feeding, there was increased number of islets, but a smaller islet per unit area which remained until day 24 (Petrik et al, 2001). We did not measure pancreatic morphology on day 12, therefore we do not know whether changes may have occurred before 18 days.

4. Fatty Acid Synthase and Plasma Lipid Concentrations

The lipogenic properties of FR are well documented (Elliott et al, 2002). Fatty acid synthase is one of the major lipogenic enzymes, and therefore contributes to TG production. Since FR is more lipogenic than glucose, and may be exacerbated by hyperlipidemia (Jeppesen et al, 1995; Elliott et al, 2002) or hyperinsulinemia (Abraha et al, 1998) we compared hepatic fatty acid synthase (FAS) activities and plasma non-esterified fatty acid (NEFA) and plasma triglyceride (TG) concentrations among the three diet groups.

Hepatocytes from MF rats expressed almost twice the FAS activity (~11 mmol/min·mg protein) compared to both RMS and FR rats (~6 mmol/min·mg protein). FR rats had the highest plasma NEFA concentrations under fed conditions and while they were approximately 20% higher than MF rats, they were 60% higher than RMS rats. No significant differences were observed in either fasting or fed-state TG concentrations among the three groups.

Researchers using the HC model have studied the effects of diet during the suckling period on the lipogenic capacity of liver as well as adipose tissues (epididymal and omental) (Hiremagalur et al, 1992). They found that HC rats expressed increases in two key lipogenic enzymes, fatty acid synthase and glucose-6-phosphate dehydrogenase, along with higher rates of lipid synthesis *in vitro*. However, these comparisons were made after weaning on to a high carbohydrate diet. We found that hepatocytes from MF rats had the highest FAS activities and no significant differences were detected between RMS and FR rats. This suggests that the AR procedure contributes to increased FAS

activity at 18 days. Continuation of a HF diet, or stress induced by the AR procedure itself may contribute to the observed effects.

Under normal circumstances, there is a gradual transition during weaning to a high carbohydrate diet (Henning, 1985) and usually begins once they open their eyes on postnatal day 12 and begin to nibble on the mother's diet. Perdereau et al (1990) have shown the concentration of mRNA as well as the activity of acetyl-CoA carboxylase and fatty acid synthase are very low in the livers of suckling rats. After weaning to a high-carbohydrate diet, the rapid increment in acetyl-CoA carboxylase and fatty acid synthase mRNA concentrations (10–20-fold) is followed by parallel changes in enzyme activities whereas, weaning to a high-fat diet prevents the increase in mRNA and activity of acetyl-CoA carboxylase and fatty acid synthase.

5. Body and Organ Weights

We found that body weights were significantly lower in AR rats over most of the study (day 10-18 for RMS rats vs day 10-15 for FR rats) compared to MF rats. However, there were no differences observed in kidney, liver, fat pad or pancreas weights expressed as an absolute value, or after controlling for differences in body weight. Differences were observed in spleen and small intestines weights after controlling for differences in body weight. Both FR and RMS rats had significantly higher spleen weights compared to MF rats. Spleen weight could be used as an indicator of gross infection, which is unlikely in the AR rats as they did not display other signs or symptoms. Anatomically, the spleen is connected to the greater curvature of the stomach by the gastrosplenic (gastrolienal) ligament and is covered by visceral peritoneum on all of its surfaces. Since AR pups

underwent gastrostomy surgeries it is possible that the difference observed in spleen weights is due to scar tissue that was difficult to distinguish and was not dissected away completely before the spleen was weighed.

RMS rats had small intestines that weighed more than small intestines from both FR and MF rats. It is unclear why the small intestines RMS rats would weigh more than small intestines from the other groups. All animals were treated during the dissection in the same way and by a skilled and experienced person. It is possible that the AR technique affects the small intestine in a way that could affect its weight and perhaps a property of the FR diet inhibits SI growth or hypertrophy.

CHAPTER 6. CONCLUSIONS AND SUMMARY

1. Conclusions

Hypotheses:

1. We expected that rats that were fed FR during suckling would have an elevated insulin response to an oral glucose challenge and would be less glucose tolerant compared to both MF and RMS rats. We showed that although were no differences in plasma insulin concentrations under fasting conditions, but there was a heightened insulin response to an oral glucose challenge. There were no differences in glucose tolerance among the three diet groups at 18 days in response to either a low dose (0.3g/kg) or high dose (3g/kg) glucose challenge. Therefore, feeding FR during the suckling period leads to an increased plasma insulin response to an oral glucose challenge but does not affect glucose tolerance.

2. We anticipated that rats fed fructose before weaning would have higher plasma levels of TG and NEFA and FAS activity than either MF or RMS rats. We found no significant differences in plasma TG concentrations in either fasting or fed conditions among the three diet groups. Plasma NEFA concentrations in FR and MF rats were higher compared to RMS rats. FAS activity was significantly lower in the AR rats compared to MF. Therefore, the AR rearing process leads to lower FAS activities in the livers of 18 day old rats. This is sufficient to suppress elevated NEFA concentrations in RMS rats, but not in FR rats and this has no effect on TG concentrations.

3. Since the diets we used were isocaloric and were similar in composition to rats' milk, we expected that rat pups in each treatment group (FR, RMS, MF) would have similar growth rates, and organ weights. We did not detect differences in body weight at 18 days although growth rates differed during the study. The AR technique can impair growth of pups due to difficulties associated with hand-rearing animals. Although most organ weights were similar, the spleens were larger in AR rats and the small intestines were larger in RMS rats. Higher spleen weights were likely due to scar tissue and it is unclear why RMS rats have heavier small intestine weights.
4. The suckling period is a critical period of development of the endocrine pancreas in the rat. We expected that FR feeding during suckling would lead to an increased ß-cell number and size when compared to RMS and MF rats. FR rats had the fewest number of β-cell clusters compared to RMS rats and MF rats. Therefore, the fewer β-cells in FR rats are producing more insulin per cell.

5. We expected that rats fed FR would have altered ß-cell number and size, and that *in vitro* and *in vivo* studies of insulin secretion would be consistent. However, we were unable to confirm elevated insulin secretion *in vivo* using *in vitro* techniques. Therefore, *in vitro* simulation of insulin secretion may not accurately reproduce the *in vivo* processes.

2. General Summary

Other researchers have examined the effects of nutritional interventions during suckling; however, no one has studied the effects of FR. This research generated some interesting findings about the possible consequences of FR feeding during a critical period for the pancreas. A number of questions arise, such as: Does the increased insulin response to an oral glucose challenge persist into adulthood? Does impaired glucose tolerance occur after weaning to a high carbohydrate diet? Can similar conclusions be made about the effects of FR introduction at young ages in humans? These questions would help us apply our understanding of the effects of FR during suckling that we observed to strategies aimed at preventing T2D diabetes in humans.

CHAPTER 7: REFERENCES

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APPENDIX A: Metamorph Procedure for Measuring Proportion of Positive Insulin Staining in Pancreas Tissue

Step 1. Find top left corner (10X) of pancreas section in field of view. Under **Acquire** tab select **Start Focusing**. Adjust picture so that there is good contrast with brown staining (exposure time sets brightness). When satisfied with picture quality, take picture of slide (**Stop Focusing**, set zoom to 50%).

Step 2. Under **Display** tab choose **Color Separate**, select **New** beside **Green** and **Blue Image** (if staining present in picture). You should have three pictures on the screen, regular, green and blue.

Step 3. Open **Calibrate Distances** dialog box under the **Measure** tab. Select Blue Image and the calibration setting for the magnification (10X).

Step 4. In the **Blue Image**, go set the threshold using the **Threshold** toolbar (on left side of image). A properly thresholded image will display the islets only and will not capture dust, refracted light or other non-islet tissue. If this is difficult, draw a region around the islets using the **Region** Toolbar.

Step 5. Under **Measure** tab, select **Integrated Morphometry Analysis (IMA)**. Choose **Open Log** feature make sure you open an Excel Spreadsheet, to ensure manageability open a new file and organize your data later. Under **Configure Log**, select **Image Name, Region Name, Object# and Area** in the Excel Spreadsheet add two columns to the right Type (your characterization of the tissue using initials) and Image (so that you can organize by picture #1 etc). Choose significant figures under **Format Settings** tab in **IMA**(#.#####; # = decimal place).

Step 6. Still working in the **IMA** panel, Select **Blue** under **Image** and **Set up Parameters** for Measuring, click box beside **Area** (will exclude white space). Under Measure choose **Display Objects**. **Reset filters** will reset the lower limit to zero and will capture any thresholded object. To exclude non-islet objects, find smallest islet in picture (when comparing to regular image) (*make sure Parameter is now changed to **Classifying** and that **Find** is selected under **Image Interactive Mode**, also Parameters need to be the same in **Measure** and **Classify**, or no data will be displayed). Once you know the area of the smallest Islet put this area in the **Filter** range. If this data is suitable select **Log Data** and then classify the islets manually in Excel and **Save** spreadsheet after each entry since it may crash.

Step 7. To measure **Non-pancreas Area** and **Pancreas Area**, open up the **Green** Image. Before thresholding image, use the **Wavy Region Tool** to draw separate regions around different tissue types. For adipose tissue draw region closely around the tissue since white space will need to be counted due to the contribution of TG to its area.

Step 8. Repeat Step 5 and 6 in **Green**. To measure by **Region**, click on the **Region** until outline is flashing to indicate proper region, then classify tissue type in excel and save.